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Timo Tamminen & Jorma Kuparinen: On the measurement of heterotrophic activity in the aquatic environment using labelled substrates
Tiivistelmä: Vesistöjen bakteeriplanktonin aktiivisuuden mittauksesta radioaktiivisten merkkiaineiden avulla 4

Timo Tamminen: Linear transformations of the Michaelis-Menten kinetic equation in natural microbial communities research
Tiivistelmä: Michaelis-Menten -kinetiikan lineaarimuunnokset luonnon mikrobiyhteisöjen tutkimuksessa 11

Timo Tamminen, Seppo Kaitala & Ludmila V. Iliash: The heterotrophic glucose uptake potential of three marine dinoflagellates
Tiivistelmä: Kolmen merellisen dinoflagellaatin glukoosinottopotentiaali 21

Jorma Kuparinen, Kirsti Lahti, Tuija Talsi, Timo Tamminen & Anneli Virtanen: Determination of the Michaelis-Menten kinetic parameters with single concentration assays
Tiivistelmä: Bakteeriplanktonin glukoosinottoa kuvaavien kineettisten parametrien määritys yhden substraattipitoisuuden menetelmällä 26

Jorma Kuparinen, Kirsti Lahti, Ari Mäkelä, Seppo Rekolainen, Tuija Talsi, Timo Tamminen, Anneli Virtanen & Antti Uusi-Rauva: A practical approach to the measurement of microbial heterotrophic activity by the single concentration method
Tiivistelmä: Vesistöjen bakteeriplanktonin aktiivisuuden määritys yhden substraattipitoisuuden menetelmällä: käytännön suoritus 35

Tuija Talsi, Timo Tamminen & Jorma Kuparinen: Variability in planktonic heterotrophic activity and primary productivity assays in relation to sampling strategies
Tiivistelmä: Perustuotannon ja bakteeriplanktonin aktiivisuuden mittauksen tilastollinen luotettavuus näytteenoton suunnittelun perustana 42

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A PRACTICAL APPROACH TO THE MEASUREMENT OF MICROBIAL HETEROTROPHIC ACTIVITY BY THE SINGLE CONCENTRATION METHOD

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The technical procedure of the single concentration method for the measurement of parameters describing microbial heterotrophic activity in the aquatic environment is described and discussed.

Index words: Heterotrophic activity, bacterioplankton, single concentration method, glucose assimilation

1. INTRODUCTION

In this series of articles on methodological aspects of the measurement of microbial heterotrophic activity in aquatic environments using labelled substrates, both multi-concentration (kinetic) and single concentration methods have been discussed (Kuparinen et al. 1984, Tamminen 1984). Both methods are based on measurements of radioactivity incorporated and/or respired by heterotrophic organisms at defined substrate concentrations. The purpose of this paper is firstly, to describe in detail how to run the single concentration assay in practice and secondly, to discuss the required conditions for sampling, sample treatment and measurement in order to achieve a result which represents the conditions prevailing in the water at the time of sampling. The questions of the precision of the method and of the validity of a single measurement for generalizations between water bodies are discussed by Talsi et al. (1984).

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2. PARAMETERS

Four parameters can be derived with the single concentration method. Three of the parameters (heterotrophic activity, 1/T, heterotrophic potential, V and respiration percentage, R %) are based on actual measurements and the fourth (concentration sum, K+S) can be calculated from two of the measured parameters.

2.1 Heterotrophic activity, 1/T

When labelled substrates are added at tracer level concentrations (less than 20 % of the concentration of the compound in the natural aquatic environment, Wright 1974, Kuparinen et al. 1984, the turnover rate (1/T) may be determined (Williams and Askew 1968, Azam and Holm-Hansen 1973). The turnover rate of the substrate (% per hour) is calculated using the equation:

$$1/T = \frac{c}{C \cdot t} \cdot 100\ [\%\ h^{-1}] \qquad (1)$$

where
1/T = turnover rate
c = radioactivity measured after incubation; incorporated and/or respired by the organisms (dpm = disintegrations per minute)
C = radioactivity added in the sample (dpm)
t = incubation time (h)

The equation gives the percentage of substrate assimilated in one hour. Organisms retain part of the labelled substrate assimilated, and another part of the label is respired as $^{14}CO_2$ or $^{3}H_2O$. Bacterial exudation is usually considered negligible. Either net assimilation (incorporation in cells), respiration (mineralization) or total uptake (incorporation + mineralization) of the substrate can thus be measured. Since substrate addition must be small, the use of high specific activity ^{3}H-substrates is recommended in this assay. This ensures reliably detectable activities in the measurements, as the specific activity of ^{3}H is two orders of magnitude higher than that of ^{14}C. If respiration is to be measured, ^{14}C-labelling must be used, as respiration measurement with ^{3}H-substrates is rather complex (Dietz and Albright 1978, Kuparinen and Tamminen 1982). Large incubation volumes and a method of collecting all the respired $^{14}CO_2$ may be necessary when working at tracer level substrate concentrations (Kadota et al. 1966, Williams and Askew 1968).

From the measurements needed for the turnover rate determination we also obtain the turnover time (T) of the substrate ($T = C \cdot t \cdot c^{-1}$). This parameter expresses the time within which the whole amount of substrate would be removed if the uptake continued unchanged and no further supply of substrate were added. The parameter is needed in order to calculate K+S (see 2.4).

2.2 Heterotrophic potential, V

When labelled substrates are added at saturation level concentrations (substantially higher than the concentration of the substrate in the natural aquatic environment, see Kuparinen et al. 1984), an approximation of the maximum uptake velocity (Wright and Hobbie 1966) may be determined:

$$V = \frac{c}{C \cdot t} \cdot A, \qquad (2)$$

where
A = substrate concentration added in the sample ($\mu g \cdot l^{-1}$)
V = heterotrophic potential ($\mu g \cdot l^{-1} \cdot h^{-1}$)

Measured radioactivity (c) can represent net assimilation, respiration or total uptake of the substrate as in heterotrophic activity assays (2.1).

2.3 Respiration percentage, R %

Respiration measurement by the technique of Kuparinen and Uusi-Rauva (1980) is based on the collection of respired $^{14}CO_2$ in a closed serum bottle containing ethanolamine (Fig. 1). The respiration percentage of the substrate is calculated from:

$$R\ \% = \frac{c_r}{c_r + c_i} \cdot 100\ [\%] \qquad (3)$$

where
c_r = measured respiration (dpm)
c_i = measured incorporation in cells (dpm)

2.4 Concentration sum. K+S

In the kinetic method, the concentration sum (K+S) can be determined from the regression line

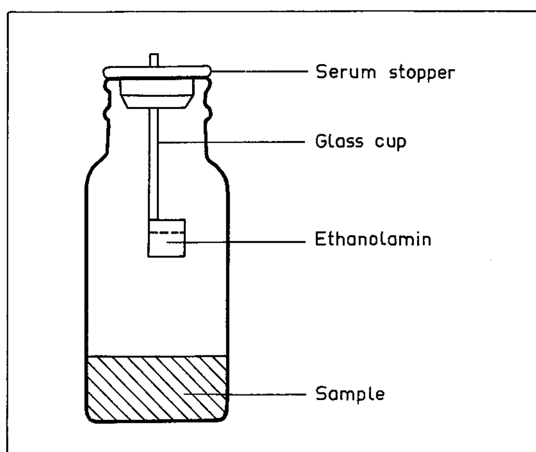


Fig. 1. Equipment for collecting respired $^{14}CO_2$.

(see Tamminen 1984). In this sum value, K is the Michaelis-Menten half-saturation constant and indicates the affinity of the population for the substrate in question. If the natural substrate concentration (S) is low, the sum value may be used to approximate substrate affinity and it thus represents an ecologically valid parameter. From the results of the single concentration assays, K+S can be calculated:

$$K + S = V \cdot T \, [\mu g \, l^{-1}] \qquad (4)$$

where
K = half-saturation constant of the substrate ($\mu g \cdot l^{-1}$)
S = concentration of the substrate in the natural aquatic environment ($\mu g \cdot l^{-1}$)
T = turnover time (h)

3. MATERIALS AND PROCEDURE

3.1 Sampling and storage

Ideally, samples should be taken aseptically. Of the commonly used Ruttner and Sormunen samplers, the latter (Fig. 3) has proved to be practical to use and easy to sterilize with alcohol. Samplers developed for microbiological work are discussed in section 4. After sampling, the samples are transferred to sterile glass bottles and stored at +4°C until further examination, unless incubated *in situ* immediately after sampling. The maximum storage time before starting the incubations is discussed in section 4.

3.2 Materials

All the glassware for sample treatment and incubation should be sterile. Oxygen or serum bottles can be used for incubation, and the latter must be used if respiration is measured according to the method of Kuparinen and Uusi-Rauva (1980). Radioactive solutions are added with a microliter pipette (e.g. Finnpipette) using sterile pipette tips.

Radioactive solutions are sterilized by filtration (0.2 μm filter porosity) or autoclaving. Filtration is recommended, since many lowweight organic molecules are easily degraded at high temperatures. If autoclaving is used, the maximum exposure is 15 minutes at 115°C. Solutions should be stored in small aliquots because re-storage of an opened ampoule is out of the question. For calculating the correct concentration levels of solutions for assays, see Kuparinen et al. (1984). The recommended substrate for routine studies is glucose (see discussion in Bölter 1981).

3.3 Incubation

Incubations should preferably be carried out *in situ* in order to minimize errors due to the effects of storage and handling. Simultaneous light and dark incubations are recommended (see Discussion). For dark incubations, black plastic tubes are practical. These should be filled with water prior to placing the incubation bottles into the tubes. Respiration measurements according to the method of Kuparinen and Uusi-Rauva (1980) must be performed in the laboratory. If *in situ* incubations are performed, some other technique for releasing $^{14}CO_2$ after incubation must be applied (Kadota et al. 1966, Williams and Askew 1968). The correct choise of incubation time is discussed in section 4.

Incubation volumes of 100 ml have generally been used for net assimilation measurements. With waters of high heterotrophic activity, smaller volumes (10 to 50 ml) are sufficient. If respiration is measured according to the method of Kuparinen and Uusi-Rauva (1980), the incubation volume is 10 ml.

Incubations are started by addition of the radioactive substrate and terminated by adding formaldehyde (35 %, 0.5 ml/100 ml of sample) Assays should be run with replicates (see Talsi et al. 1984) and a blank sample must be prepared for each assay by adding formaldehyde to one subsample before addition of the radioactivity. The impulses (dpm) of the blank sample are substracted from the means of the replicates before calculating

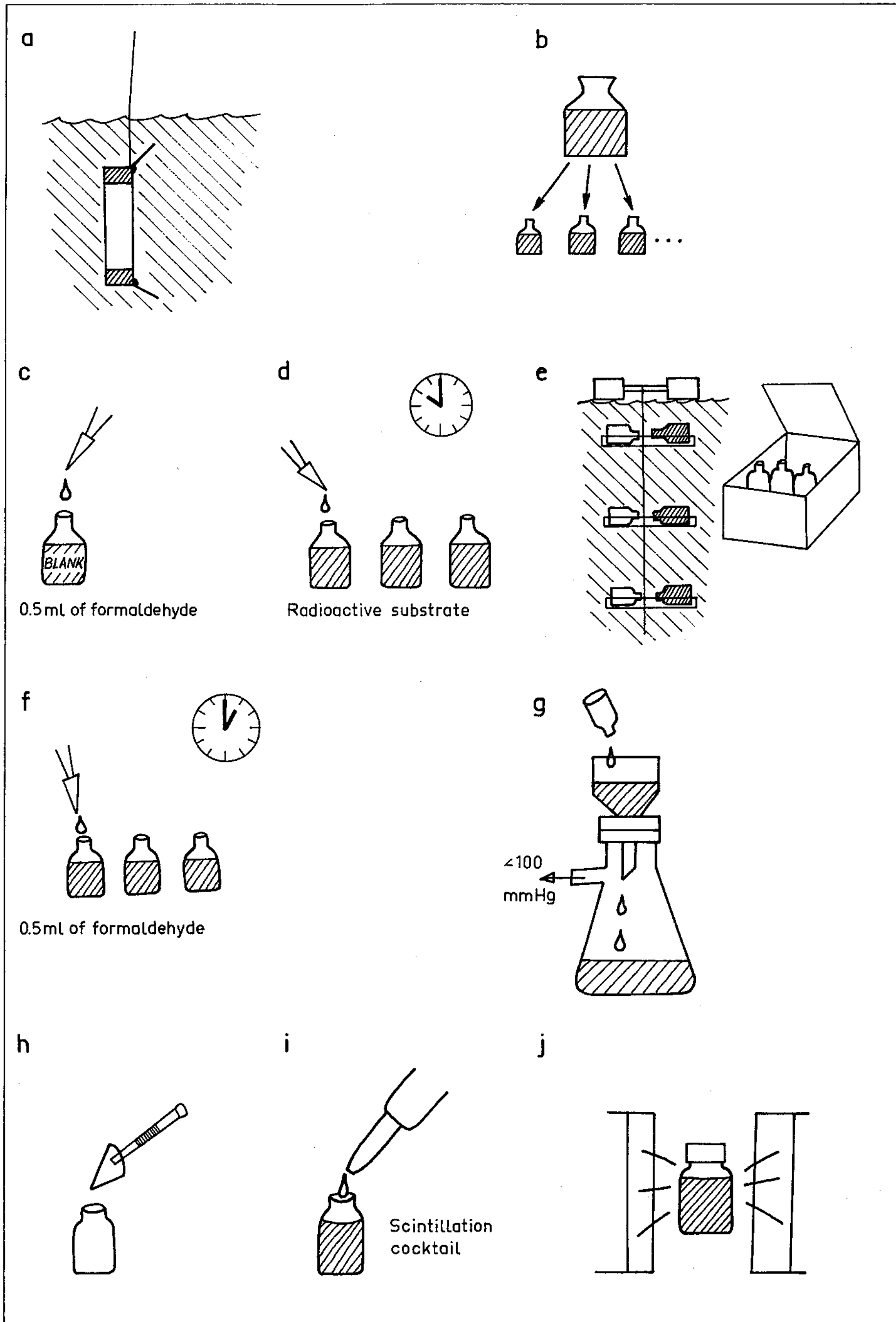


Fig. 3. The procedure for net assimilation measurements. a) sampling, b) sample is divided into replicates, c) blank is prepared by adding formaldehyde (35 %, 0.5 ml/100 ml of sample), d) radioactive substrate addition, e) incubation in the light and dark *in situ*, or in the laboratory in the dark and at *in situ* temperature, f) addition of formaldehyde, g) filtration and washing of the filter under mild vacuum (< 100 mmHg), h) placing the folded filter into a scintillation vial, i) addition of scintillation cocktail, j) measurement in a liquid scintillation counter.

the parameter values. In respiration measurements according to the method of Kuparinen and Uusi-Rauva (1980), termination is effected using 0.2 ml 4.0 M H_2SO_4, added through the serum stopper, after which ethanolamine is injected into the glass cup. After 24 hours, the respired $^{14}CO_2$ has been absorbed into the ethanolamine and the water sample is ready for filtration.

3.4 Preparation of samples for liquid scintillation counting

The glass cup containing ethanolamine is placed into a scintillation vial containing 10 ml of scintillation cocktail (5.0 g of PPO and 0.1 g of POPOP per litre of toluene) and 5.0 ml of ethanol (Kuparinen and Uusi-Rauva 1980).

Samples for net assimilation measurements are filtered on membrane filters of 0.2 μm porosity (e.g. Gelman, Millipore, Sartorius) after incubation. The filters should be washed after filtration with at least 20 ml of water having a salinity equal to that of the sample, after which they may be stored prior to counting. Storage in a freezer is recommended to prevent further microbial growth on the filter by contaminants.

Filters can be either dissolved in suitable scintillation cocktails or combusted with an oxidizer. The principal compustion and dissolving techniques for the preparation of filtered algal samples have been presented and discussed by Niemi et al. (1983) and are also applicable to bacterial samples. The results presented in Figure 2 are in good agreement with the findings of Niemi et al. (1983). It is likely that other dissolving techniques presented by Niemi et al. (1983) would also be successful with bacteria, as was the dioxan-PCS cocktail used in this work (Fig. 2). Because of the carcinogenity of dioxan, the scintillation cocktail consisting of 10 ml of PCS (Amersham International Limited, England), 0.2 ml of water and the filter is recommended. This cocktail requires at least 24 hours storage at room temperature and vigorous shaking prior to counting.

In the combustion technique with a Junitek Oxidizer (Junitek Co., Turku, Finland), the scintillation cocktail consisting of 8.0 ml of Lumasorb II and 8.0 ml of Carboluma (Lumac System, A.G., the Netherlands) was used for ^{14}C determinations, and 2.6 ml of H_2O and 7.4 ml of PCS for ^3H measurements.

For converting the cpm's to dpm's the external standard channel ratio method should be applied if low counts are expected. Otherwise the sample channel ratio method is also applicable.

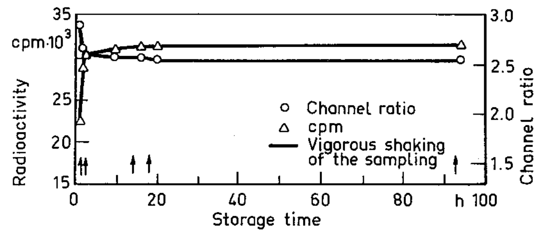


Fig. 2. Release of radioactivity (cpm, counts per minute) from ^3H-labelled bacteria on a membrane filter (Millipore) in a scintillation cocktail of 0.3 ml H_2O, 2.0 ml of dioxan and 10.0 ml of PCS (Amersham) during storage at room temperature. Channel ratios are calculated by the external standard channel ratio method.

3.5 The procedure

The basic procedure of the assay is presented in Fig. 3. If respiration is measured, refer to the previous section for details of the procedure.

4. DISCUSSION

The single concentration method for measurement of heterotrophic activity is technically analoguous with the primary productivity measurement and is therefore easy to carry out. Talsi et al. (1984) discussed the potential reliability of results obtained from a single sample and the level of variation encountered at one sampling site. The data is based on a large number of assays (over 1200 samples), and it shows clearly that the method is satisfactory because subsample variation is usually below 10 %.

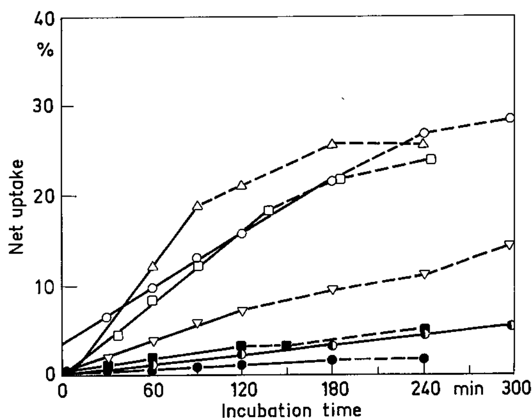
In the determination of microbiological parameters, aseptic techniques are obligatory if long incubation times (days) in enriched media are used. In routine sampling from water bodies, the requirements of aseptic procedure are often difficult to fulfill. Väätänen (1979) developed a practical sampler for aseptic surface water sampling, which may be modificated for greater depths by replacing the glass bottle with a material that tolerates hydrostatic pressure.

Sterilization with alcohol is the only practical possibility with the conventional samplers (Rutt-

ner and Sormunen samplers). These samplers may, to a neglible extent, carry organisms from surface layers to other depths on the walls of the sampler. However, the present authors question the necessity of absolute asepticity in sampling for heterotrophic activity determinations. Incubation times are usually only a few hours, and samples are exposed immediately for *in situ* incubations, or within a few hours of sampling in the laboratory. During this short incubation period it is unlikely that contaminants possibly introduced with the sampler could compete with the organisms in the sample water, which are adapted to the *in situ* temperature and to the very dilute media of natural water.

Perhaps the most serious source of error is caused by the time lag between sampling and incubation. Samples are exposed to light, pressure and temperature conditions differing from those in the original environment. Also, the so-called wall effect causes unnatural succession within the microbial community. Our data from different studies are contradictory: sometimes *in situ* values are higher than *in vitro,* sometimes vice versa. The maximum acceptable storage time of the sample before the start of incubation is dependent on the level of activity of the community of microorganisms in the sample. The shortest measured turnover times for glucose in our data were around 5 hours (see Talsi et al. 1984). In the literature, values as low as one hour have been presented (e.g. Berman et al. 1979). If the storage time exceeds the turnover time, there is a serious danger that the state of the sample may be markedly changed. Most of the heterotrophic activity data show turnover times of tens and hundreds of hours. Hence, overnight storage might be acceptable during periods of low activity. High heterotrophic activity is usually accociated with algal blooms, high temperatures and sites of allochtonous organic or nutrient enrichment. Lowest activities occur during winter and simultaneously with the algal summer minimum. Vertically, maximum activities are usually measured from the surface layers.

The correct choice of incubation time is important. It has been recommended to adjust the time so that no more than 5 % of the added radioactivity is taken up during incubation, because the scarcity of the labelled substrate produces errors (Wright and Hobbie 1966). This can be seen in long incubation times (Fig. 4). On the other hand, short incubations (a few hours) are not always possible in waters having low heterotrophic activity, especially if respiration is measured, since a reliable level of radioactivity for



■ Kaskinen 1978, ^{14}C, station 33
○ Kaskinen 1979, ^{3}H, station 35 + 1 % waste
▽ Kaskinen 1979, ^{3}H, station 26
● Kaskinen 1980, ^{3}H, station 26
□ Tvärminne 1980, ^{14}C, Storgadden
△ Raahe 1981, ^{3}H
◐ Gulf of Finland 1979, ^{14}C, LL 7

Fig. 4. Relationship between incubation time (minutes) and net uptake of the substrate (% of added radioactivity). Values are based on three or more replicates. The dotted line shows deviation from linearity.

counting must be obtained. With ^{3}H-labelled substrates, incubation times of 2 to 4 hours are usually sufficient.

The close interaction between algal and bacterial communities causes significant differences in light and dark incubations (see Tamminen 1983 for discussion). The association is largely based on algal excretion of organic substrates during active photosynthesis. Therefore, unnatural changes in light conditions during storage or incubation may cause artefacts in heterotrophic activity measurements. With *in situ* research, it is thus recommended to perform light or simultaneous light and dark incubations in order to obtain representative results for the water body. In the laboratory, dark incubations are preferred, because artefacts caused by standard illumination are likely to be significant.

LOPPUTIIVISTELMÄ

Artikkelissa kuvataan yksityiskohtaisesti nk. yhden lisäyksen menetelmän käytännön suoritus vesistön heterotrofisen aktiivisuuden mittauksissa.

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