

Determination of Kinetic Constants for Nitrifying Bacteria in Mixed Culture, with the Aid of an Electronic Computer

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SUMMARY

An electronic computer was used to integrate differential equations for bacterial growth, and thus to determine simultaneously growth-rate constants, saturation ('Michaelis') constants, and initial numbers of bacteria. In one case numerical methods were necessary to effect the integration and the work would have been prohibitively great if a computer had not been used. By this means curves have been fitted to experimental data on the course of nitrification in water from the Thames Estuary.

Kinetic constants for *Nitrosomonas* and *Nitrobacter* at various temperatures were obtained, and the rate of death of these bacteria in the absence of their substrates was estimated.

INTRODUCTION

The activities of ammonia-oxidizing and nitrite-oxidizing bacteria are important in determining both the quality of effluents from biological purification processes for the treatment of domestic and industrial wastes, and the condition of streams, rivers, and estuaries into which such effluents are discharged. To describe quantitatively the influence of these nitrifying organisms in such systems it is necessary to know their growth-constants under the relevant environmental conditions, and the saturation (Michaelis) constants of the substrates which support their growth. It is also desirable to be able to estimate the concentrations of these organisms present in a given sample. A technique for obtaining such information has been developed. This consists essentially in observing the course of nitrification during aeration of appropriate samples when incubated under constant conditions, and then the determination, by trial-and-error, of the values of the kinetic constants and initial concentration of organisms which best fit the observed data. The labour involved would be prohibitive if a desk calculator were used; possibly mainly for this reason the present approach has not received much attention hitherto, but by using an electronic computer most of this labour is eliminated. The method is illustrated below by reference to an examination of the kinetics of nitrification in samples of water from the Thames Estuary.

In the present work it has been convenient to assume that the oxidation of ammonia and nitrite are brought about, respectively, only by *Nitrosomonas* species and by *Nitrobacter* species. Nevertheless the existence of at least one other type of nitrifying micro-organism, the ammonia-oxidizing marine organism *Nitrocystis*, is reasonably well established, and since it is possible that this or other types were active in the samples we examined, the observed growth constants may in fact

represent an average for a mixed population. Since, however, the main object of this work was to develop a general technique for analysis of data from batch cultures, and since for the study of nitrification in rivers, estuaries and treatment plants information on the combined nitrifying ability of the natural population was in itself of sufficient interest to justify the work, the above simplification was considered adequate. A more complete investigation, however, would necessarily involve identifying the types and strains of organisms present.

Kinetics of growth of nitrifying bacteria

The kinetics of growth of both organisms (*Nitrosomonas*, *Nitrobacter*) appear to conform fairly closely to the following equation of the Michaelis type, as do those of various heterotrophic bacteria (Monod, 1942; Hinshelwood, 1946; Novick & Szilard, 1950; Herbert, Elsworth & Telling, 1956):

$$dC_m/dt = k_m C_m x / (x + X). \quad (1)$$

In this context C_m is the concentration of *Nitrosomonas* in mg./l., as dry matter, k_m its growth-rate constant, x the concentration and X the saturation (or Michaelis) constant of the substrate ammonia, both in mg./l. as nitrogen. References to mass and concentration of ammonia, nitrite and nitrate relate throughout to mg. nitrogen and mg. nitrogen per litre respectively. Mass and concentration of bacteria are expressed as dry wt. bacteria (dried at 105°). The corresponding equation for the growth of *Nitrobacter* is

$$dC_b/dt = k_b C_b y / (y + Y), \quad (2)$$

in which y is the existing concentration and Y the saturation constant of nitrite, C_b is the concentration and k_b the growth constant of *Nitrobacter*.

It would perhaps be surprising if this relation exactly represented bacterial growth and it should be mentioned that modifications have been proposed. For example, Boon & Laudelout (1962) included an extra term in the equation for *Nitrobacter* to allow for a slight inhibitory effect of the substrate nitrite. However, at the concentrations of nitrite found in the present experiments this term has a negligible effect.

Oxidation of ammonia. If the oxidation of unit mass of ammonia-N produces a dry mass E_m of *Nitrosomonas* organisms then

$$C_m - C_{m0} = E_m(x_0 - x), \quad (3)$$

in which x is the concentration of ammonia-N, C_m the concentration of *Nitrosomonas*, and C_{m0} and x_0 are the initial values of C_m and x . Combining equations (3) and (1) gives a differential equation that can be integrated to a form already published (Downing, Painter & Knowles, 1964), which gives the relation between concentration of ammonia-N and time:

$$k_m t = (1/A) [E_m X \log_e(x_0/x) + (A + E_m X) \log_e \{(A - E_m X)/C_{m0}\}], \quad (4)$$

where

$$A = C_{m0} + E_m x_0.$$

Solutions to this equation, calculated by using an electronic digital computer, were matched against the observations, described later, in order to determine the best values for k_m , C_{m0} and X , the programme for the machine being so devised that trial values for these variables could be chosen over as wide a range as desired. The yield

constant, E_m , was taken as 0.05, this choice being perhaps a little low on evidence quoted by Downing *et al.* (1964) and also on unpublished work at this Laboratory (Painter & Loveless, in preparation). An incorrect value for E_m would, however, affect only the estimate of C_{m0} , but not those of k_m and X . The estimated value of C_{m0} is in fact proportional to the assumed value of E_m . This can be seen from equation (4), which remains unaltered if C_{m0} and E_m are multiplied by the same factor. Figure 1(a) shows an example of the agreement between the observed and calculated curves when the values of k_m , C_{m0} and X have been properly chosen.

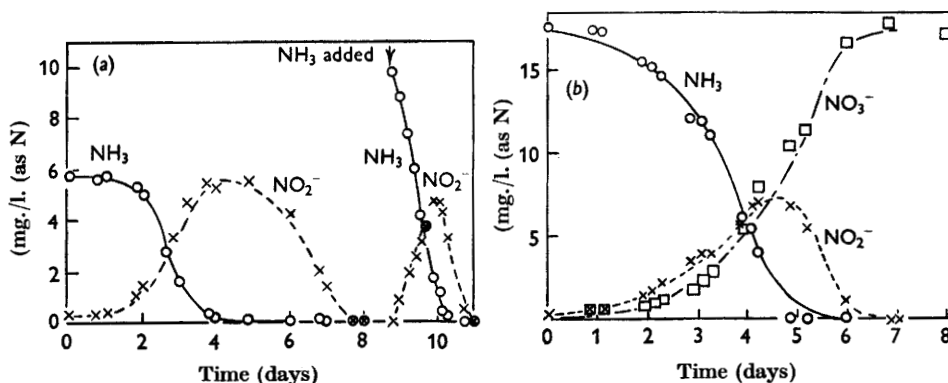


Fig. 1. Examples of computed curves which best fit observations on the course of nitrification during incubation of water from the Thames Estuary. (a) An experiment in Series 2, Table 1. Temperature 29.3° until day 8, 30.8° thereafter; more ammonia was added on day 9. (b) An experiment in Series 4, Table 1. O, ammonia observed; ×, nitrite observed; □, nitrate observed; —, ammonia computed; - - -, nitrite computed; ····, nitrate computed.

One of the problems was to decide whether or not any set of values of k_m , C_{m0} and X , giving an apparently satisfactory fit to the observed data, represented a unique solution or whether some other combination of these variables would have given an equally good fit. This problem was, however, made easier by the fact that the value of X appears to be quite low and hardly affected the shape of the ammonia-time curve at concentrations of ammonia-N above about 1 mg./l.

The idea that any combination of values which gave a good fit would be unique was supported by the fact that a second such combination which fitted equally well was never obtained, and also by the general consistency of the results, both among themselves and also as compared with other published data.

Apart from such general support, it can be shown from equation (1) (which after substituting $-E_m dx/dt$ for dC_m/dt gives the slope, dx/dt , of the ammonia-time curves) that only the correct value of k_m will give a curve passing through two experimental points, provided that the first of these points was so chosen that (a) the concentration of ammonia oxidized since the beginning of the experiment was sufficient to make C_m large compared with C_{m0} and (b) X had been correctly chosen or was small compared with x . (For any particular value of x , condition (a) ensured that all curves through one point applied to substantially the same concentration of *Nitrosomonas* and condition (b) that all such curves had substantially the same value for $x/(x+X)$.) Suppose, for instance, the correct curve was drawn through two

such points, and that k_m was of value k , then any other curve through the points must, somewhere between them, have a greater slope than the first curve, and somewhere else have a smaller slope. Where the curve has a greater slope than the correct curve at the same ammonia concentration then equation (1) shows it has a k_m value *greater* than k , for by equation (3) and condition (a) above C_m is the same for both

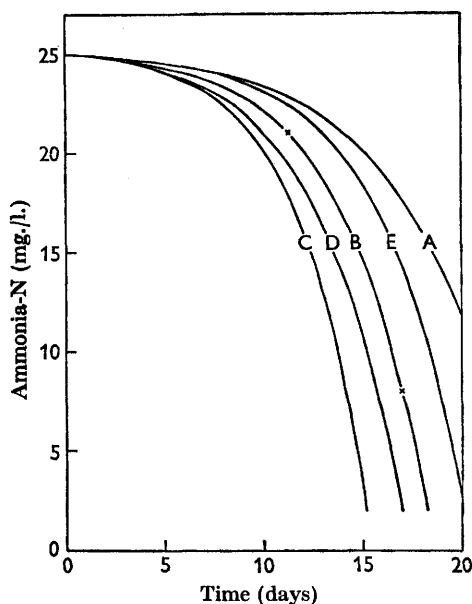


Fig. 2

Fig. 2. Effects of values of growth constant and initial concentration of *Nitrosomonas* on shape and position of computed ammonia-time curves.

A)	Growth constant	0.20	day ⁻¹ ; initial concentration of <i>Nitrosomonas</i>	0.016 mg./l.
B)		0.25		0.016 mg./l.
C)		0.30		0.016 mg./l.
D)		0.25		0.022 mg./l.
E)		0.25		0.010 mg./l.

Saturation constant, 0.36 mg./l. in each case; x, points referred to in text under the heading 'Oxidation of ammonia'.

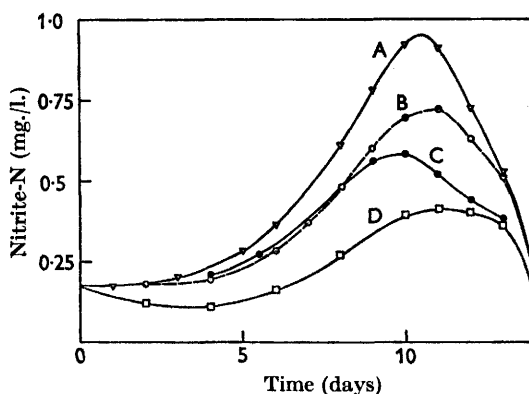


Fig. 3

Fig. 3. Effects of initial concentration and saturation constant of *Nitrobacter* on shape and position of computed nitrite-time curves.

A)	Initial concentration	0.001	mg./l.; saturation constant	2 mg./l. N
B)		0.002		2 mg./l. N
C)		0.001		1.5 mg./l. N
D)		0.004		1.5 mg./l. N

Growth constant 2 days⁻¹ in each case.

curves, and by condition (b) so is $x/(x+X)$. Similarly, where the curve has a smaller slope it has a k_m value *smaller* than k . Therefore it does not have a constant value for k_m and so cannot be a solution. Such points, in Fig. 2, are 21 mg./l. at 11.3 days and 8 mg./l. at 17 days; only curve B goes through both points. Thus, when a fit is obtained to a number of points along an appropriate part of the ammonia-time curve it is very probable that the correct k_m value has been used. Having established

a value of k_m by the middle part of an ammonia-time curve then an argument similar to the above shows that the values of X obtained from the fit to the later part of the curve are correct, subject to the accuracy of the determinations of low concentrations of ammonia.

For estimating C_{m0} , the initial concentration of Nitrosomonas, it is assumed that no lag-phase intervened between taking the sample and continuance of growth with a steady growth-rate constant. This assumption is made on the ground that, except when the incubation temperature was different from that in the estuary, the bacteria probably did not necessarily undergo any great change in their chemical environment or physical conditions on being transferred from the estuary to the sample bottle. When the incubation temperature was low the changes in concentration of ammonia in the early stages were too small to provide evidence of a lag-phase. There is certainly no indication of such a lag where the incubation temperature was of the order of 30° , since the concentration of ammonia decreased rapidly from zero time, giving an experimental curve that can be well fitted throughout by equation (4), using fixed values of k_m , C_{m0} and X . When in any experiment a lag-phase did occur, the effect would be that the estimate of C_{m0} would be too low, but that the estimate of k_m , essentially based on fitting those parts of the experimental curve which show definite curvature, would be correct. In passing it may be noted that while only one set of values of k_m , C_{m0} and X would genuinely fit perfect data, the precision with which these values can be selected is, of course, in practice dependent on the accuracy of the observations.

Oxidation of nitrite. In extending the calculations to the changes in concentration of nitrite with time the concentration of Nitrobacter is given by an equation like equation (3), viz.:

$$C_b - C_{b0} = E_b \{y_0 + f_m(x_0 - x) - y\}, \quad (5)$$

where y is the concentration of nitrite-N, C_b the concentration of Nitrobacter, C_{b0} and y_0 are the initial values of C_b and y , f_m is the ratio of the mass of nitrite-N formed to that of ammonia-N oxidized, and where the oxidation of unit mass of nitrite-N produces a dry mass E_b of Nitrobacter.

The approximate constancy of the total amount of inorganic nitrogen present throughout the period of incubation (as shown, for example, in Fig. 1*b*) indicates that the value of f_m is close to unity; it has been taken as 0.99 in the present calculations.

Substituting the value of C_m given by equation (3) into equation (1) we then have a differential equation for the variation of ammonia-N with time, which may be summarized as

$$dx/dt = \phi_1(C_{m0}, x_0, x, X, k_m, E_m), \quad (6)$$

the integrated form of which is equation (4). Similarly, substituting the value of C_b given by equation (5) in equation (2) gives a differential equation for the variation of nitrite-N with time, summarized as

$$dy/dt = \phi_2(C_{b0}, x, y_0, y, Y, k_b, E_b). \quad (7)$$

$\phi_1()$ and $\phi_2()$ are general expressions for functions. The occurrence of x in the expression for dy/dt , together with the involved relation between x and t given in equation (4), makes it impossible to integrate equation (7) to an explicit relation between nitrite concentration, y , and time, t . Accordingly equation (7) was treated

by 'numerical integration' to obtain a predicted curve for concentration of nitrite against time for any chosen set of values for C_{b0} , k_b , and Y . From the evidence quoted by Downing *et al.* (1964) E_b , the mass of Nitrobacter formed per unit mass of nitrite-N oxidized, was taken as 0.02; choice of a wrong value for E_b would affect the estimation of the initial concentration of bacteria (the result being proportional to the value chosen for E_b) but would not affect the estimates of growth-rate and saturation constants.

By combining differential equations (6) and (7) with a 'subroutine' based on the Runge-Kutta-Merson procedure (Fox, 1962), and supplied by the makers of the digital computer used, a single program was formed, which gave predicted curves for many sets of C_{b0} , k_b , and Y at the rate of about one every 2 min. (with a desk calculator about 20 hr was required for each curve). The values used for C_{m0} , k_m and X were those that had been found, as described earlier, to give the best fit to the ammonia-time curve.

The availability of such a numerical integration 'subroutine' is of great assistance in writing a programme for solving simultaneous differential equations. Makers of computers are usually able to supply a version of the Runge-Kutta process programmed in a language suitable for their machines, while for workers using the 'universal' computer language, Algol, a programme published by Lukehart (1963) for the Runge-Kutta-Merson method is suitable.

In this way were determined values of C_{b0} , k_b and Y for Nitrobacter that gave the best fit to the experimental points; examples of such best fits are shown in Fig. 1. From the many trials made, it seemed that, as for Nitrosomonas, a good fit could be obtained only with a particular set of values of these three unknowns except in one or two cases in which a completely satisfactory fit could not be obtained with any combination. The predicted curves were much altered by variation in any of the unknowns, the nature of the change depending on the unknown concerned. For example, in Fig. 3 the change from A to C is produced by decrease of the saturation constant from 2 to 1.5 mg./l., from A to B by increasing the assumed initial concentration of Nitrobacter from 0.001 to 0.002 mg./l., and from C to D by increasing this from 0.001 to 0.004 mg./l. As in the case of Nitrosomonas, when a lag-phase were present the estimates of C_{b0} , the initial concentration of Nitrobacter, would be too low.

The prediction of the change in concentration of nitrate with time was made from the best fit kinetic constants and values of C_{m0} and C_{b0} , assuming that unit mass of nitrite-N forms 0.99 unit mass of nitrate-N on oxidation. This assumption appears to be justified by the close fit so obtained, as illustrated for example in Fig. 1*b*.

METHODS

Five series of experiments were made, the objectives being to study the way in which the kinetic constants of nitrifying bacteria were affected by temperature, by aeration in the absence of the energy source (ammonia or nitrite) and by concentration of dissolved oxygen, and to examine the possibility that a relation exists between estimated concentrations of nitrifying bacteria in Thames water and the concentration of suspended matter. The first four series were made in 1960 and the fifth in 1963.

Series 1. Subsamples from 50 (British Imperial) gallons (about 227 l.) of water,

from the Thames Estuary near London Bridge, were incubated, without dilution, in 10 l. aspirators in three constant-temperature rooms at about 7.5°, 14° and 20°. Two experiments were made in each room, one with the water as taken, and the other with the addition of ammonium sulphate to raise the initial concentration of ammonia-N by about 6 mg./l. Solids present in each sample were kept in suspension with magnetic stirrers. Each aspirator was completely filled and the mouth of the vessel closed by a rubber stopper, care being taken to exclude air when the stopper was inserted. Samples were generally taken daily from each vessel by removing the stopper and allowing water to flow through a side arm near the bottom. Concentrations of dissolved oxygen, $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$ and total oxidized-N ($\text{NO}_2\text{-N}$ plus $\text{NO}_3\text{-N}$) were measured in each sample.

Complete filling of the aspirator was not essential for the aspects now under discussion but was done for the purpose of measuring the rate of consumption of dissolved oxygen. When necessary the contents of the aspirator were aerated by shaking in contact with air, an additional small sample being taken for determination of dissolved oxygen. The aspirator was then replenished from another that was open to the air and contained a further portion of the original sample of Thames water. In calculating the results of the experiments, allowance was made for the slightly different composition of the replacement water. This allowance was a very small one because the analyses for ammonia, nitrite and nitrate gave similar figures for aspirator contents and replenishment water (which indicated similar growth of nitrifying bacteria), and especially because the volume of water used per day for replenishment was only about 2% of the total volume of the sample. From these considerations it was clear that the replenishment would not significantly change either the concentration of the nitrifying bacteria or the general biological conditions.

The same analytical methods were used in all series of experiments.

Ammonia-N was determined by distillation of the sample from a borax buffer solution, nesslerization of the distillate, and measurement of the extinction in a Spekker absorptiometer.

Total oxidized-N was determined by reduction of a 50 ml. sample with Devarda's alloy in the presence of 5 ml. of 8% (w/v) sodium hydroxide solution, followed by distillation and then nesslerization of a suitable sample of the distillate. A weighed amount (1 g.) of Devarda's alloy was used, and a blank determination was made for each series of analyses.

$\text{NO}_2\text{-N}$ was measured by using Griess-Ilosvay reagent according to the procedure recommended by the Ministry of Housing and Local Government (1956).

Dissolved oxygen was measured by the azide modification of the Winkler method, as recommended by the Ministry of Housing and Local Government (1956).

Series 2. Thames water containing nearly 3 mg./l. ammonia-N (taken from about the same position as the water used in Series 1) was incubated without dilution, at about 28° in three vessels with the addition of ammonium sulphate equivalent to 0, 2.5, and 5 mg./l. ammonia-N. On the ninth day (by which time nitrification was complete) a further quantity of ammonium sulphate was added to each vessel to give concentrations of 2, 10, and 20 mg./l. ammonia-N, respectively, and the incubation continued. The techniques used in Series 1 were again used, except that the oxygen content of the water was replenished as necessary by bubbling oxygen through it for short intervals to give initial concentrations of 12–15 mg./l.

Series 3 and 4. It was evident from the results of the tests made in Series 1 and 2 that the quantity of nitrifying bacteria in the Thames water was fairly small, so before starting Series 3 and 4, in which the effect of the concentration of dissolved oxygen on the nitrification process was examined, the sample of Thames water was stored at 19° (the temperature of the subsequent incubation) for several days to obtain a larger population of nitrifying organisms by the oxidation of the ammonia-N present initially. The water was then fortified with ammonium sulphate equivalent to 18 mg./l. ammonia-N, and incubated in six vessels. A different oxygen content was maintained in each vessel by aerating with mixtures of air, nitrogen, and carbon dioxide. The rate of flow of each gas was measured by a rotameter, the total rate of flow being about the same in each vessel. The partial pressure of the carbon dioxide in the gas mixture was adjusted to a value that would maintain the concentration of free carbon dioxide that was present in the water at the beginning of the experiment. The required rate was obtained in a subsidiary experiment by adjusting the rate of flow to maintain the initial pH value of the water. Subsequent experiments, and work reported elsewhere (*Department of Scientific and Industrial Research*, 1964) indicate that loss of ammonia by volatilization during the experiments was unlikely to be of significant proportions.

Series 5. In these experiments the progress of nitrification was measured in a series of five samples taken from the Thames Estuary at London Bridge on the same day and from the same depth but at different states of tide; the concentration of suspended matter in the samples ranged from about 100 to 1000 mg./l., and the object was to examine whether or not the initial concentration of nitrifying bacteria bore any relation to that of suspended solids. It was thought that such a relation might exist if nitrifying bacteria became bound up in the estuary with suspended mud particles, which tend to be flocculent in nature, and that since the concentration of suspended mud varied with the state of the tide so too might that of the nitrifying bacteria; however, no significant relation was found. The procedure was similar to that in the experiments of Series 3 and 4, except that normal air was used for aeration.

RESULTS

Table 1 gives details of the results of the five series of tests.

Growth constants

Effect of temperature. It will be seen from Table 1 and also from Figs. 4 and 5 that the growth constants of the organisms present in Sample 1 increased considerably with increasing temperature in the range from 8 to 23°. The equation of the regression line fitted to the data for *Nitrosomonas* is

$$\log_{10} k_m = 0.0413T - 0.944$$

(with k_m in day⁻¹ and T in °C.), the implication of which is that the growth-rate constant increased by about 9.5 % of the existing value per degree Centigrade increase in temperature. The corresponding equation for *Nitrobacter* is

$$\log_{10} k_b = 0.0255T - 0.492$$

(with k_b in day⁻¹ and T in °C.), implying that the temperature coefficient for this organism was about 5.9 %/deg. It is also evident that at a given temperature the value

Table 1. *Growth-rate constants, saturation constants and initial concentrations for Nitrosomonas and Nitrobacter in samples from estuary of River Thames*

Series no. and date of sampling	Original sample no.	Temper- ature of incu- bation (°)	Chloride (Cl- mg./l.)	Sus- pended solids (mg./l.)	Dissolved oxygen during incubation (mg./l.)			Range of pH values	Initial ammonia- N (mg./l.)	Nitrosomonas			Nitrobacter		
					Min.	Average	Max.			k_m (day ⁻¹)	\bar{X} NH ₃ -N (mg./l.)	C_{50} (mg.*./l.)	k_b (day ⁻¹)	\bar{Y} NH ₃ -N (mg./l.)	C_{50} (mg.*./l.)
1; 11 Jan. 1960	1	8.3	280	69	1.3	5.6	9.7	7.5-7.6	8.0	0.2	0.2	0.0028	0.5	0.2	0.0008
	1	8.6	280	67	1.3	5.3	8.3	7.5-7.7	2.7	0.27	0.2	0.001	0.6	0.18	0.00022
	1	13.9	280	74	1.4	5.5	9.4	7.4-7.6	8.5	0.45	0.4	0.0013	0.7	0.18	0.0003
	1	14.5	280	67	2.3	5.9	9.2	7.5	2.7	0.58	0.4	0.0004	0.7	0.25	0.0006
	1	22.2	280	68	1.0	4.9	7.5	7.6-7.7	2.7	1.02	1.0	0.002	1.2	1.0	0.0013
2; 28 Mar. 1960	1	23.2	280	71	0.5	4.2	7.6	7.7-7.8	8.2	0.85	1.2	0.003	1.3	1.3	0.0008
	2	28.5	—	125	1.2	5.5	11.9	7.4-7.7	2.9	2.0	1.1	0.00065	2.0	2.0	0.0001
	2	29.2	—	125	1.5	10.4	18.0	—	1.7	1.8	1.8	0.085	1.5	3.0	0.05
	2	29.3	—	143	1.0	5.6	11.4	7.7	5.7	1.95	2.0	0.003	2.0	3.0	0.0002
	2	30.8	—	143	2.3	8.4	14.6	—	9.8	1.9	2.0	0.10	2.0	7.0	0.10
3; 8 Nov. 1960	2	28.8	—	137	1.6	5.6	11.0	7.7	8.3	2.0	1.6	0.0025	1.9	5.0	0.0005
	2	29.8	—	137	2.0	8.2	19.0	—	19.6	2.1	8.0	0.17	2.5	8.0	0.15
	3	19.0	32	—	8.1	8.3	8.4	7.3	17.2	0.7	0.6	0.055	1.2	2.0	0.03
	3	19.0	32	—	6.5	6.6	6.8	—	17.2	0.68	0.6	0.05	1.2	1.5	0.02
	3	19.0	32	—	4.4	5.1	5.9	—	17.3	0.65	0.6	0.088	1.1	1.5	0.01
4; 6 Dec. 1960	3	19.0	32	—	2.6	3.4	4.0	—	17.7	0.75	0.6	0.06	1.0	1.7	0.015
	3	19.0	32	—	1.4	2.2	2.6	—	17.7	0.57	0.6	0.11	0.7	1.6	0.016
	4	18.8	33	—	7.6	8.4	8.8	—	18.0	0.7	0.6	0.015	0.9	1.5	0.02
	4	18.8	33	—	4.0	4.7	4.9	—	17.9	0.7	0.6	0.049	1.1	1.7	0.01
	4	18.8	33	—	3.2	3.4	3.8	—	17.4	0.7	0.6	0.04	0.8	1.7	0.03
5; 9 Sept. 1963	4	18.8	33	—	1.9	2.0	2.3	7.4-8.6	17.8	0.7	0.6	0.044	0.9	2.0	0.01
	4	18.8	33	—	1.2	1.4	1.9	—	17.6	0.6	0.6	0.026	0.67	1.9	0.012
	4	18.8	33	—	0.5	0.6	0.7	—	17.8	0.5	3.0	0.04	0.6	2.5	0.035
	5	27.0	—	103	7.9	7.9	7.9	—	18.9	1.5	1.7	0.017	—	—	—
	6	27.0	—	253	7.9	7.9	7.9	—	19.1	1.5	1.7	0.017	—	—	—
7; 27.0	7	27.0	—	980	7.9	7.9	7.9	—	19.3	1.5	1.7	0.017	—	—	—
	8	27.0	—	750	7.9	7.9	7.9	—	19.5	1.5	1.7	0.019	—	—	—
	9	27.0	—	470	7.9	7.9	7.9	—	19.7	1.5	1.7	0.017	—	—	—

* Mg. bacteria after drying at 105°.

of the growth-rate constant for *Nitrobacter* was about 50 % greater than that for *Nitrosomonas*. Data for the remaining Samples 2–5 appear to be in good agreement with the trends of those from Sample 1.

Buswell, Shiota, Lawrence & van Meter (1954) studied the effect of temperature on the growth of pure cultures of *Nitrosomonas* and obtained values of k_m increasing from about 0.5 day⁻¹ at 15° to about 2 days⁻¹ at 32°; the temperature coefficient

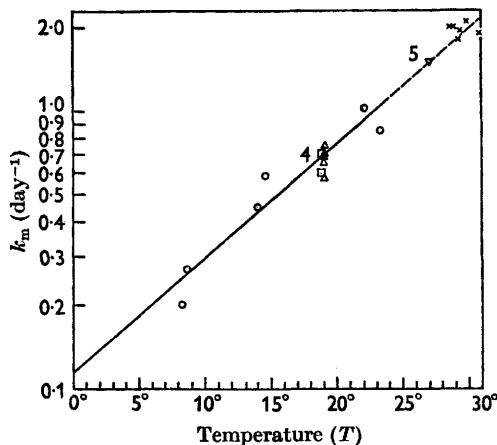


Fig. 4

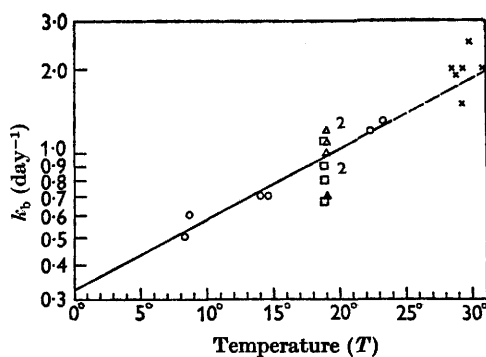


Fig. 5

Fig. 4. Effect of temperature on the growth-rate constant for *Nitrosomonas*. Regression line is fitted to the points for Sample 1, Table 1. ○, Sample 1; ×, Sample 2; △, Sample 3; □, Sample 4; ▽, Sample 5. A number alongside a point shows the number of determinations giving a value represented by that single point.

Fig. 5. Effect of temperature on the growth-rate constant for *Nitrobacter*. Regression line is fitted to the points for Sample 1, Table 1. ○, Sample 1; ×, Sample 2; △, Sample 3; □, Sample 4. A number alongside a point shows the number of determinations giving a value represented by that single point.

calculated from these data is 8.2 %/deg. A similar effect of temperature on the growth of *Nitrosomonas* in activated sludge from a sewage-treatment plant was reported by Downing *et al.* (1964) and by Downing & Hopwood (1965). The magnitude of k_m at a given temperature is known to depend on the pH value of the medium and its composition. At temperatures of about 30°, for which k_m in the present work ranged from about 1.6 to 2.0 days⁻¹, the following values (all in days⁻¹) were reported for pure batch cultures grown in medium containing mineral solids: 1.19 (Engel, 1980), 1.03 (Bömeke, 1946), 0.46 (Lees, 1952), and 1.5 (Engel & Alexander, 1958). Skinner & Walker (1961) obtained a figure of 3.1 days⁻¹ during continuous culture, with a clear medium of composition adjusted to give maximum growth rates. Painter & Loveless (unpublished work in this Laboratory) obtained maximum values of k_m of about 0.7 to 0.9 day⁻¹ at 25° and pH 7.6–8.0 in batch cultures in a clear medium; beyond this range they found that growth rates decreased markedly. For comparison with the growth-rate constants found for *Nitrobacter* there appears to be only a figure of 1.89 day⁻¹ at 32° obtained by Boon & Laudelout (1962) with a pure culture; this is only about half the corresponding value in the present work.

Figure 1 is a semi-logarithmic plot showing the yield of $\text{NO}_2\text{-N}$ (mg./l.) versus Temperature (T) in degrees Celsius. The y-axis is logarithmic, ranging from 0.1 to 10. The x-axis is linear, ranging from 0 to 30. Data points are plotted as open circles, and a solid line represents the fitted curve. The data points show an increasing trend, with some scatter at higher temperatures.

Temperature (T)	Yield ($\text{NO}_2\text{-N}$ as mg./l.)
8.5	0.18
14.5	0.18
15.5	0.25
18.5	1.0
19.5	1.0
20.5	1.0
21.5	0.9
22.5	1.2
28.5	2.5
29.5	4.0
29.5	6.0
30.5	8.0
30.5	9.0

Fig. 7. Effect of temperature on saturation constant for *Nitrobacter*. Regression line is fitted to all points, [Samples 1 to 5, Table 1. A number alongside a point shows the number of determinations giving a value represented by that single point.

Saturation constants

As regards the saturation constant of ammonia-N for *Nitrosomonas* there is little to afford a comparison with the values shown in Fig. 6, other than a value of 0.2 mg./l. at 21° obtained at this Laboratory by Dr H. A. Painter (but this was for activated sludge from a sewage-treatment plant) and an approximate value of

10 mg./l. at 30° calculated by Downing *et al.* (1964) from data by Hofman & Lees (1953). Accordingly, Fig. 6 appears to be the first evidence for a large temperature coefficient of the saturation constant for ammonia (*Nitrosomonas*). The equation of the regression line in Fig. 6 is

$$\log_{10} X = 0.051T - 1.158 \quad (8)$$

(with X in mg./l. N and T in °C.); this corresponds to an increase in saturation constant of about 11.8% of the existing value per degree Centigrade increase in temperature.

The saturation constants found for nitrite-N are in good agreement with the figure for pure culture of *Nitrobacter* (6 mg./l. at 30°) calculated by Downing *et al.* (1964) from the data of Lees & Simpson (1954), and of 8.4 mg./l. at 32° found by Laudelout & van Tichelen (1960), though the latter is different from the value of 22 mg./l. at 32° given by Boon & Laudelout (1962). Laudelout & van Tichelen found an appreciable temperature coefficient (though not so large as that determined in the present experiments), their values being 1.4 mg./l. at 14°, 2.1 mg./l. at 18°, 8.4 mg./l. at 32°. The equation of the regression line fitted to our values in Fig. 7 is

$$\log_{10} Y = 0.063T - 1.149 \quad (9)$$

(with Y in mg./l. N and T in °C.); this corresponds to a temperature coefficient of about 14.5% per degree.

The actual values of X and Y (particularly X) for Series 4, last subsample, in which the concentration of dissolved oxygen was maintained at a very low value, are higher than expected from the results on other subsamples from the same and other series; this may represent not a true change in X and Y but the existence, in the growth-rate equation, of a term containing dissolved-oxygen concentration but having a significant effect on growth rate only at low concentrations of dissolved oxygen.

Initial concentrations, C_{m0} , C_{b0}

It will be seen from Table 1 that for each separate group of subsamples derived from Samples 1 to 4 there was considerable variation in the estimated initial concentrations of nitrifying bacteria, but whereas the average values for each series varied by about a factor of 30, the range of variation of the estimates within each group was between limits only of about 2 and 0.5 times the mean value (neglecting one very low figure in the group of subsamples derived from Sample 1); furthermore the ratio of the mean values of C_{b0} to those of C_{m0} for each series varied between comparatively narrow limits of 0.30 and 0.68. The second, fourth and sixth subsamples of Sample 2 showed high values of C_{m0} and C_{b0} because they had already undergone one period of incubation (see earlier).

It is not yet known whether the observed variability in the values of C_{m0} and C_{b0} in some groups of subsamples was due to a variable lag-phase or to real variations, resulting perhaps from a failure to subdivide reproducibly the suspended matter in the original sample. Thus the question of the accuracy of the estimates must remain in doubt. On the other hand, the degree of variation is not particularly high and there seem to be no definite grounds for believing that the values were necessarily greatly in error.

At present it is not possible to offer any explanation of the large differences in the

Table 2. *Effect of absence of substrate on apparent concentrations of Nitrosomonas and Nitrobacter remaining viable during aeration of water from the Thames Estuary*

Temperature of incubation (°)	1st phase				2nd phase		
	Initial concentration Nitrosomonas (mg.*/l.)	Ammonia-N oxidized (mg./l.)	Nitrosomonas formed (mg.*/l.)	Days without ammonia	Expected initial concentration Nitrosomonas (mg.*/l.)	Actual initial concentration Nitrosomonas (mg.*/l.)	Nitrosomonas survival (%)
Nitrosomonas							
29	0.00065	2.9	0.15	4.4	0.15	0.085†	57†
29	0.0025	8.3	0.42	5.6	0.42	0.17	40
30	0.003	5.7	0.29	4.8	0.29	0.1	34
Temperature of incubation (°)	Initial concentration Nitrobacter (mg.*/l.)	Nitrite-N oxidized (mg./l.)	Nitrobacter formed (mg.*/l.)	Days without nitrite	Expected initial concentration Nitrobacter (mg.*/l.)	Actual initial concentration Nitrobacter (mg.*/l.)	Nitrobacter survival (%)
Nitrobacter							
29	0.0001	3.15	0.063	0.6	0.063	0.05†	79†
29	0.0005	8.6	0.172	0.8	0.172	0.15	87
30	0.0002	5.95	0.12	0.8	0.12	0.1	83

* Mg. bacteria after drying at 105°.

† Values for this sample approximate owing to scatter of experimental points.

initial concentrations of nitrifying micro-organisms estimated to be present in samples taken from the estuary at different times, other than those due to pre-aeration of the samples, to which reference has already been made. On the other hand, it would not be surprising if such large variations did occur. Quite apart from the fact that the estuary is far from completely mixed, it would appear that, as indicated below, the organisms did not survive for long periods in the absence of substrate. Moreover, the population density at a given place was also considerably dependent on the concentrations of ammonia, nitrite, and dissolved oxygen, and the variations in these along the estuary. All these factors change markedly with freshwater flow, temperature, and tidal conditions.

Survival of nitrifying organisms. In the case of the subsamples from Sample 2 to which further ammonia was added a day or two after all ammonia originally present had been completely oxidized to nitrate, it appears from data summarized in Table 2 that in this second phase, although the growth constant for both organisms remained substantially unchanged, a good fit to the experimental data could be obtained only by assuming initial numbers of nitrifying bacteria much lower than those calculated to have been formed from the oxidation of the ammonia originally present. Thus for one sample (observed and calculated curves for which are shown in Fig. 1*b* and other data in Table 2) the concentration of *Nitrosomonas* at zero days was estimated at 0.003 mg./l. From the concentration of ammonia oxidized this would have increased to 0.29 mg./l. at 5 days, but at the start of the second part of the experiment (8.8 days) the concentration of *Nitrosomonas* was estimated to be only 0.10 mg./l. (E_m was 0.05 for these calculations but assumption of any other value, E , would merely multiply each estimated value by $E/0.05$). It thus appears that a substantial amount of *Nitrosomonas* may have died during the period when dissolved oxygen was present but ammonia was not; two other experiments of the same kind, results of which are included in Table 2, lead to the same conclusion. Although the data are somewhat variable, the proportion of *Nitrobacter* apparently surviving during the interval when nitrite was absent, was higher than the corresponding proportion of *Nitrosomonas*; possibly this is connected with the fact that this period was much shorter than that for which the *Nitrosomonas* was deprived of substrate (ammonia).

DISCUSSION

The work shows the usefulness of electronic computers for the rapid integration of differential equations for bacterial growth, particularly those which describe sequential growth of interdependent bacteria and which can only be solved numerically. By these means, estimates of kinetic constants and initial concentrations of bacteria can be obtained quite rapidly from experimental data about the changes in concentration of substrates and products with time in batch cultures. Application of the same method to sequences of enzyme-catalysed reactions could no doubt be made for the purpose of obtaining the velocity-constant for each stage.

The quite accurate fitting of the curves for variations with time in concentrations of ammonia and nitrite in incubated samples containing nitrifying bacteria (assumed to be species of *Nitrosomonas* and *Nitrobacter*), using the Michaelis type of growth-rate equation, is a further example of the applicability of this type of relation to bacterial growth. Originally developed for enzymic reactions, for which it is widely successful, the Michaelis rate-equation is theoretically based on the idea of reversible

formation of any enzyme + substrate complex. It is perhaps not surprising that this rate-equation, developed for enzymic reactions in homogeneous solution, applies well to *Nitrobacter*, in which the nitrite-oxidizing system may be almost in the external medium possibly being situated, according to Boon & Laudelout (1962), near or at the outer surface of the cell-wall. The ammonia-oxidizing system of *Nitrosomonas* (involving two stages, the first yielding hydroxylamine) may be similarly situated; if, on the other hand, it is located within the cell, then it can be shown (Rashevsky, 1960) that the Michaelis form of rate-equation would still apply, provided that the rate of transport of ammonia by simple diffusion through the cell-wall to the interior is so high that the concentration of ammonia at the site of reaction is little below that in the external medium. Even this condition need not be met if, instead, transport through the cell-wall is by formation of an intermediate complex or compound, as shown to be the case for phosphate transport into *Staphylococcus aureus*, for which a Michaelis type expression represents the rate of transport (Mitchell, 1959). Transport by formation of an intermediate complex appears to apply also to amino acids entering *Escherichia coli* (Britten & McClure, 1962) and glucose absorption by mammalian cells (see Hober, 1947), and has been suggested for calcium, potassium and sodium entering mammalian cells (Hasselbach, 1962).

The results of the present work support data reported by Buswell *et al.* (1954) indicating a large temperature coefficient for the growth-rate constant for *Nitrosomonas*, and show, further, that the saturation constant of ammonia for this organism is somewhat similarly dependent on temperature. The results also show a similar relation between growth-rate constant and temperature for *Nitrobacter* and confirm qualitatively the previously reported high temperature coefficient for the saturation constant for *Nitrobacter*. It would appear from the present work that both types of organism tend to die fairly rapidly when aerated in the absence of the relevant energy source. The growth-rate constants of the surviving bacteria were, however, little changed by such treatment, in agreement with Buswell *et al.* (1954) who showed that it was necessary to keep *Nitrosomonas* aerated for 12 days without ammonia to produce an alteration in growth-rate constant.

Mr A. L. H. Gameson was responsible for planning the sampling and experimental work in 1960; most of the determinations at that time were made by Mr C. G. Ogden. Sampling and determinations in the 1963 work were done by Mr L. J. Scragg.

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