Death rate during the exponential growth of *E. coli*

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*Version of 10 December 2021
Very provisional. Comments are welcome.*

Key-words: Escherichia coli, cell division, mortality rate, exponential phase, stationary phase

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Abstract

So fast is the growth of a culture of *E. coli* that it led researchers to overlook a possible death rate. As a matter of fact, the experiments done in the first half of the 20th century were unable to detect any mortality. It is only at the beginning of the 21st century that positive evidence emerged which confirmed the existence of a sizeable (albeit small) mortality. In the present paper this mortality was measured at successive 45mn time intervals from early exponential growth into the stationary phase. Done with a flow cytometer, the successive measurements of the ratio dead/living also provided an estimate of the standard deviation of the mortality rate. In a forthcoming experiment we are planning to focus more closely on the stationary phase in order to find out if its mortality rate is lower or higher than in the exponential phase.
1 Introduction

1.1 Reasons for measuring the mortality rate of bacteria

In any population, whether human or non-human, the birth and death rates are the two most basic variables which define its evolution. Historically, these were the first variables which could be measured annually (i.e. independently of the decennial censuses). One should not think it was an easy task. For instance, in the United states, in spite of the fact that the earliest data, namely those for Massachusetts, go back to the 19th century, it is only around 1930 that the so-called birth and death “Registration Areas” were able to cover all the states. Basic as they are, these rates nevertheless continue to raise unsolved questions. Here are two examples.

Regarding birth rates, successive prediction attempts put foward in past decades regularly proved wrong by a wide margin (of 15% or more).

For death rates the situation is different because the causes of death are well known which means that, in contrast to birth rates, there are no unexplained fluctuations. What remains mysterious however is the overall pattern of death rates as a function of age. Over the age of 15 years, the death rate follows the well defined empirical law discovered by Benjamin Gompertz in the 19th century (Gompertz 1825). This law states that the death rate is an exponential function of age; basically it doubles when age increases by 10 years. For ages under 15 there is also a well-defined law; it states that the death rate $\mu$ decreases with age like an hyperbola function: $\mu \sim 1/t^\alpha$, where $t$ is the age and $\alpha$ an exponent which is close to 1. In previous studies (Berrut et al. 2016, Bois et al. 2019) we have shown that this law also holds for non-human multicellular organisms. It is of great interest to see if it holds also for unicellular organisms for the following reason. In multicellular organisms one can distinguish separate organs, with death usually occurring when one or several crucial organ fail. In bacteria the situation is less clear. Thus, this test may give us information on the internal organization of unicellular organisms. However, before turning to age-specific death rates we wish to give fairly accurate estimates of the global death rate.

1.2 Outline of the paper

The paper proceeds as follows.

- In the next section we describe the methods and results obtained in previous studies.
- In section 3 we carefully discuss and define the death rate of a population of bacteria.
- In section 4 we explain the protocol of our measurements, we present our results and we discuss how their accuracy can be improved.
In the last section we discuss related perspectives.

2 Previous studies of death rates of bacteria in exponential growth

The investigation of the death rate of bacteria in their phase of exponential growth has been going on for over a century. This long-lasting sustained interest underlines that the question is of importance. At the same time the fact that it is not yet completely solved suggests that this measurement still represents an experimental challenge. That is mostly due to the fact that the death rate is small. Nowadays we know that for *E. coli* the death rate is of the order of 1 per 1,000 living cells and per hour.

In order to identify the trend of the death rate across the exponential phase and in the early stationary phase requires an even higher accuracy because the error bars must be smaller than the effect of the trend itself.

Actually the very definition of the death rate is not obvious. Even if one leaves apart the question of the biological meaning of the notion of “death”, it is not obvious to adapt standard demographic notions to a population which doubles every 20mn.

In the following subsections it will be seen that there are basically three methods for distinguishing dead from living cells.

- The CFU (Colony Forming Unit) method. The fact that this traditional method does not have the required accuracy is clearly shown in Wilson (1922), a paper which will be discussed in more details below.

- The second method consists in following all individual cells belonging to one cohort over as many generations as possible. Introduced in 1932, this method was vastly improved in a paper of 2005 by Stewart et al.

- Finally, the third method is based on Flow Cytometry (FC). It is this method that will be used in the present paper.

2.1 The Wilson paper (1922)

In his paper Wilson called into question the dogma that in a young broth culture (up to 24 hours) all bacteria are living. Here is what he writes at the beginning of his paper.

“On looking up the literature it was found that of the many observers [the author cites 9 papers published between 1898 and 1920] who had made a comparison of the two counts [namely the total number of cells that can be counted in a culture, whether dead or alive on the one hand and the number of viable cells defined as cells that are able to fission on the other hand],
the discrepancy was passed over with little comment. Any discrepancy was attributed to “errors.”

In a 41-page long paper the author examines one by one all successive operations required in counting procedures. He tries to make them as rigorous as possible and he estimates the remaining error margin. For instance dilution before counting is an operation which requires great care but nevertheless cannot be made very precise. In fact, the author was facing an impossible task. Such global counting techniques were beset by too many uncertainties. Therefore, it is not surprising that Wilson arrives at vague and disappointing conclusions (p.444).

“It seems that in cultures of Bact. suipesifer there is a normal death rate even during the period of maximum growth. Its extent will vary from culture to culture. In some it is as high as 43%, in others it is only 20% or 10%, while finally in a few it is for a short period actually nil.

2.2 Paper of Kelly and Rahn (1932)

The first lines of the paper provide a clear statement of why this is a key-issue. “It has been assumed by many bacteriologists that during the period of rapid growth, in a satisfactory culture medium, some bacteria will die in spite of good food and favorable environment. No doubt this assumption was derived from an analogy with populations of higher forms of life, of which a number of individuals are known to die before they reach the reproductive age even with good care.” (Kelly et al. 1932, p.147)

The observation of individual cells pioneered by Kelly and Rahn represented a breakthrough. It is true that they were unable to see any death in the exponential phase but that was only because their sample of 1,766 cells was too small. Their methodology was sound and opened the door to further observations with larger samples. Yet, this did not happen until 73 years later. Indeed, in 2005 this investigation was resumed. Thanks to modern computerized counting techniques and a sample some 20 times larger namely 35,000 E. coli cells, the experiment revealed some 16 deaths; see Stewart et al. (2005) and the discussion below.

Using a method pioneered by J. Orskov (1922), Kelly and Rahn followed the replication of individual bacteria (and one yeast species) on a solid medium. They recorded the family trees spanning 4 generations. Altogether they observed 1,766 divisions: 977 of Bacterium aerogenes, 325 of Bacillus cereus, 464 Saccharomyces ellipsoideus. On average the time intervals between fissions of Bacterium aerogenes was 30 mn but with great inter-individual fluctuations (coefficient of variation $\sigma/m$ close to 100%).
Table 1: Landmark papers about bacterial death rates in the exponential phase

| Year | Paper          | Method | Sample size | Number of deaths | Death rate (dr) per 1,000 and per hour |
|------|----------------|--------|-------------|------------------|--------------------------------------|
| 1    | 1922 Wilson    | Global | several sets| unreliable       | unreliable                           |
| 2    | 1932 Kelly (1) | Individual | 733         | < 1              | dr < 4.1                             |
| 3    | 1932 Kelly (2) | Individual | 420         | < 1              | dr < 1.4                             |
| 4    | 2005 Stewart   | Individual | 35,049      | 16               | 1.5                                  |
| 5    | 2008 Fontaine  | Global  | 1,000,000   | 700              | 0.7                                  |

Notes: “Global” means measurement performed on a large number of cells in liquid medium. For this global measurement it is the technique of flow cytometry which brought about a breakthrough and allowed reliable measurements. The following bacteria and yeast were investigated (in parenthesis is the length in minutes of the reproduction cycle). 1: Bact. suipesfifer and other species, 2: Bacterium aerogenes (30mn), 3: Saccharomyces ellipoideus (105mn), 4: E. coli (30mn), 5: E. coli (30mn). Sources: Based on the papers cited in the third column.

The observations led the authors to the following conclusions.
- There was not a single instance where a cell became dormant.
- In the very words used by the author, there was not a single case of “infant mortality”, that is to say a cell which died after division.

The first conclusion is reassuring because, even today, researchers often worry about the risk of mistaking dormant cells for dead cells (see the discussion at the beginning of Garvey et al. 2007).

The second conclusion is also interesting for it shows that if such an infant mortality exists (as is indeed observed in experiments done in the past two decades as reported below) then its rate is lower than: \( \frac{1}{1766} = 0.56 \) per 1,000. (for a time interval of 30mn). This is a rough estimate obtained by bulking together all three species. Estimates detailed by species (at least for the two largest) are given in Table 1.

Whereas the measurement of Stewart et al. (2005) relies on the observation of individual cells, a paper of 2008 by Fontaine et al. relies on a global (not individual) observation. It is to the discussion of these modern investigations that we turn now.

2.3 The paper by Stewart et al. (2005)

In the Kelly and Rahn (1932) experiment successive divisions were followed over 4 generations, a process which from each single initial cell produced \( 2^4 = 16 \) cells. In the Stewart et al (2005) experiment up to 9 generations were followed, a process through which each initial cell gave rise to \( 2^9 = 512 \) E. coli cells. Time-lapse images were taken and analyzed automatically thanks to dedicated software. As 94 colonies were analyzed this led to a total of 35,049 divisions.
The criterion used for the definition of death was immobility combined with no division. Some 16 cell death were observed. Unfortunately their sizes were not included in the publication because the main purpose of the paper was a different issue.

2.4 Paper of 2008 by Fontaine et al. (FC measurement)

In the technique that is used here the cells are not monitored individually. Instead, the recourse to flow cytometry allows global estimates. Stained dead cells, or more precisely those cells whose breached membranes allow the stain to drift into the cytoplasm, are counted thanks to a flow cytometer (FC). In such a device the light of a laser is diffused by the cells when they move through the beam, then received by a sensor and amplified by a photomultiplier and finally analyzed by a computer software algorithm. Flow cytometry allows many characteristics of the cells to be identified and recorded. Here this technique is used to count stained or fluorescent dead cells.

Flow cytometry began to be used in the 1950s and was really a game changer. It replaced the successive manipulations needed in the CFU method.

Although FC is the key of the measurement method, a number of additional verification tests are required to ensure that what is measured by the device is indeed the appropriate death rate.

3 Definition of the global death rate of bacteria

In previously published mortality data for bacteria (for instance in Steward et al. 2005) their real meaning and definition, for instance with respect to the time interval, was not clearly indicated. That is why we discuss this point in some detail.

First, we recall the standard definition of the death rate in human demography. Then we examine how this definition should be adapted to apply to organisms whose life duration is of the order of 20 mn.

3.1 Global death rates in human populations

In Appendix A we recall the reasons which lead to the definition of the death rate as accepted in human demography.

Consider a population of size \( x \) that one observes during a time interval \( \Delta t \). If \( \Delta y \) denotes the number of deaths during \( \Delta t \), the death rate in this interval is

\[
\frac{\Delta y}{\Delta t} = \frac{\text{number of deaths}}{\text{time interval}}
\]
time interval will be defined by:

\[ \mu(t) = 1000 \left( \frac{1}{\Delta t} \right) \frac{\Delta y}{x(t)} \]  

(1)

where: \( x(t) \) denotes the size of the population.

If \( \Delta t \) is expressed in a time unit \( u \), the unit of \( \mu(t) \) will be “per 1,000 individuals and per \( u \)”.  

For a human population \( x(t) \) can be measured at the beginning, mid-point or end of the one-year observation time because it changes little over one year. The situation is very different when one is dealing with a population whose size doubles every 20mn. In the next subsection it will be seen that cell numbers should rather be replaced by life cycles.

As an example of how to use the previous definition let us assume a population of one million in which 120 deaths are counted in the month of January. If we take years as unit of time, the interval of observation will be: \( \Delta t = 1/12 \); thus, the January death rate will be \( \mu = 1000 \times 12 \times 120 \times 10^{-6} = 1.44 \) per 1,000 individuals and per year.

### 3.2 Two special requirements for a population of bacteria

For bacteria formula (1) must be applied with special care. Why?

Strictly speaking, \( x(t) \) should not be the whole population existing at time \( t \) but only the individuals who are subject to the risk of death during the time interval \( \Delta t \). For instance, if one wishes to compute the death rate of the French population in year 2000 one should not take for \( x(t) \) the whole population alive on 31 December 2000 for this population would include individuals born on the last day of 2000 and who are subject to the risk of dying for only one day. Clearly, the number of such individuals is negligible compared to the whole population and one can therefore forget this restriction. However, with a population which doubles every 20mn this restriction must be taken into account. We will see below how to do that.

There is another meaningful difference. To explain it, let us consider a fictitious human population in which all individuals live 100 years. We assume further that the initial population is 1 million and that it remains constant with births strictly compensating the deaths. Now, suppose we wish to estimate the death rate over a period \( \Delta t \) of 300 years. This is basically the situation we are facing with a sample of \( E. coli \), except that here we assumed the population to be constant for the sake of simplicity. In applying formula (1) should we take \( x(t) \) equal to 1 million? That would clearly be incorrect, for during the 300 years there were 3 generations successively exposed to the risk of dying, each producing a number of dead individuals which will be
counted cumulatively. In other words, instead of taking for \( x(t) \) 1 million one should take the number of life cycles which is 3 millions. Therefore, when applying formula (1) to \( E. coli \) one must also take for \( x(t) \) the number of life cycles which occurred during the interval of observation \( \Delta t \).

The number of life cycles for a population growing exponentially is computed in Appendix A.

### 3.3 Global death rate of bacteria: modeling method

The previous method required several assumptions to be made. Can one devise a more direct argument?

The present modeling method relies on two simple equations which describe a population in exponential growth. Any population can be fully described by two parameters: (i) the birth rate and (ii) the death rate \( \mu \). In order to estimate them one needs to connect them to two variables that can be measured in our experiment.

For the birth rate we can start from the fact that the population grows exponentially: \( x(t) = x_0 \exp(\alpha t) \). The exponent \( \alpha \) can be readily obtained from the data of the spectrophotometer. It is true that this device makes no difference between the cells which are alive and those that are dead, but the second are about 1,000 times less numerous than the first and can therefore be neglected.

The cytometer provides the ratio \( y(t)/x(t) \) where \( x(t) \) represents the number of living cells and \( y(t) \) the number of dead cells. We have already written an evolution equation for \( x(t) \), can we also write one for \( y(t) \)? The evolution equation results from the definition (1) written in differential form:

\[
\mu = \frac{1}{dt} \frac{dy}{x(t)} \rightarrow \frac{dy}{dt} = \mu x(t) = \mu x_0 \exp(\alpha t)
\]

If one assumes that \( \alpha \) is a constant, this equation can be solved easily and one gets a relationship between \( x(t) \) and \( y(t) \):

\[
y(t) - y_0 = \frac{\mu x_0}{\alpha} \exp(\alpha t) + K \quad \rightarrow \quad y(t) - y_0 = \frac{\mu}{\alpha} [x(t) - x_0]
\]

where \( x_0, y_0 \) are the initial values at the start of the 45mn time intervals between successive measurements.

This equation implies that if \( x(t) \) growth exponentially, then, so does \( y(t) \); therefore, the initial values can be neglected in comparison to the values taken by \( x(t), y(t) \) some 45mn later. \( x_0 \ll x(t = 45 \text{ mn}) \) and \( y_0 \ll y(t = 45 \text{ mn}) \). Thus, the relationship between \( x(t) \) and \( y(t) \) can be written in the simpler form:

\[
\mu = \frac{y(t)}{x(t)^\alpha} \quad \text{where} \quad \alpha = \log[x(t)/x_0]/t \quad (3)
\]
In practice, the ratio \( x(t)/x_0 \) can be read on the graph of the optical density. Just to see what is the order of magnitude of the death rate \( \mu \), we take an \( \alpha \) corresponding to a doubling time of 25mn which is the average in our experiment. \( \alpha = \log(2)/0.42 = 1.66 \text{ h}^{-1} \)

This gives:

\[
\mu = 1000 \times 1.66 \frac{y(t)}{x(t)}
\]

Five measurements of the ratio \( y(t)/x(t) \) covering the first 3 hours of the exponential phase, gave the following average: \( < y(t)/x(t) > = 0.0011 \pm 0.00022 \) which in turn leads to the following expression of the death rate:

\[
\mu = 1.83 \pm 0.20 \text{ per 1,000 individuals and per hour}
\]

## 4 Methodology of the experiment

The following description includes two parts that are often omitted but which are of great importance for the success of our experiment. The first is the selection of the micro-organism best suited for our investigation. The second is the list of preliminary tests performed to check for possible sources of errors in the successive steps of the measurement process. By listing these sources of fluctuations our hope is also that it may help to improve the accuracy of the observation.

### 4.1 Design of the experiment: selection of an appropriate strain

As our objective is well defined we need to select the micro-organisms which are best suited. There are several requirements that we discuss below and which are summarized in Table 2.

1. The main requirement is a short division time so that one could get a large population in a fairly short time.

2. After measuring the overall death rate over a life cycle, in the second part of this investigation, we are planning to measure the age-specific death rate which means that we need a way to estimate the individual age of each cell. For rod-like organisms experiencing transversal fission the bacteria length provides a possible proxy for its age. The characteristics of diverse model organisms summarized in Table 1 show that *Escherichia coli, Bacillus subtilis* and *Paramecium caudatum* are three possible candidates. However, if one wishes to count the dead cells with a cytometer, the last two should be discarded because they may clog up the thin pipes of the cytometer.
Table 2 Characteristics of unicellular organisms suitable for mortality studies

| Name                  | Shape    | Fission type | Division time (mn, hour) | Size (micro-m) | Chain formation | Prokaryot or Eukaryot |
|-----------------------|----------|--------------|--------------------------|----------------|-----------------|-----------------------|
| *E. coli*             | rod      | transversal  | 20mn                     | 1              | little          | P                     |
| *Bacillus subtilis*   | rod      | transversal  | 20mn                     | 1              | yes             | P                     |
| *Saccharomices cerevisae* | round    | budding      | 2h                       | 3              | yes             | E                     |
| *Euglena gracilis*    | elastic  | longitudinal | 2h                       | 20 − 100       | yes             | E                     |
| *Paramecium caudatum* | long     | transversal  | 20h                       | 100            | no              | E                     |

(Paramecium should anyway be discarded due to its long generation time). This leaves us with *E. coli*.

3 Another consequence of pipe narrowness is the fact that one cannot use highly concentrated cultures. In addition such cultures would not be suitable because at the end of their exponential phase. Therefore, if one wishes to test millions of bacteria (in order to get several thousands of deaths) one must use a cytometer which accepts a large sample volume.

### 4.2 Organization of the experiment

The successive steps of the experiment follow quite naturally from the end objective. They are summarized in Fig.1. Every 45mn, a sample was extracted from the culture which was growing in the incubator. Then, (i) its OD was measured (ii) the sample was diluted with PBS so as to have in each trial the same cell concentration (iii) Propidium iodide was added (iv) 1ml of the sample was introduced in the cytometer.

In terms of time intervals,
- It took about 10mn from sample extraction to the PI step.
- 15mn were given to the dead cells for absorbing the PI stain. Note that the absorption is faster when the concentration of the stain is higher. The PI is said to be toxic but it would require a separate assay to estimate the concentration level above which the toxicity effect becomes notable.
- The treatment of the 1ml sample in the FC took about 10mn to 15mn. In fact, the value of the ratio dead/total observed after 2 or 3mn remained very stable in the remaining time. This means that a smaller volume would have given the same results.
Fig. 1 Organization of the experiment. The two main devices are the spectrophotometer which measures the optical density (which in turn gives a measure of the number of cells per cubic cm) and the flow cytometer which counts the number of dead cells after they have been made fluorescent by absorption of propidium iodide. Note that the time interval between extraction of a sample from the culture and its introduction in the cytometer is about 20 mn.

4.3 Preliminary tests

Our tests concerned mainly three points.

- When does the stationary phase really begin? The transition becomes clearly visible toward an optical density of 0.3 (for a more elaborate discussion see Sezonof et al. 2007). However, by starting a culture with a concentration of cells some 1,000 smaller than the smallest OD that the spectrophotometer could measure, we have seen that there is in fact a steady increase of the doubling time.

- In order to test the accuracy of the identification of the dead cell we prepared a mixture 50%–50% of dead cells (killed by a temperature shock) and living cells. A proportion of 45% dead cells was detected by the FC. As a complementary test, the dead cells sorted by the FC were reintroduced into the FC. A ratio dead/total of 97% was observed.

- In an attempt to better understand the connection between “PI death” and “immobility death”, dead cells sorted by the FC were put under a microscope equipped with a light source in the fluorescence range of the PI. It was verified that there remained no moving cells in the sample. This test tells us that our “PI dead” would also be counted as dead with the immobility criterion used in the experiment of Stewart et al. (2005). In other words, PI death is either equivalent to or “deeper” than “immobility death”\(^1\).

\(^1\)During an observation of the embryonic growth of eggs of Zebra fish, one embryo remained completely motionless during two days. It was thought to be dead but in fact resumed a normal development. Compared with normal embryos, the only difference was that this one hatched two days later.
5 Results

5.1 Absolute versus relative measurements

It must be stated from the outset that we are more interested in relative than in absolute values of the death rate. There are two main reasons for that.

- The absolute value of the death rate is dependent upon the criterion used to define “death”. We have already mentioned that PI death seems to be a sharper criterion than death defined by immobility. In addition, even within PI death the technical definition of death relies on how one selects the lower limit of PI positive events. On Fig.2 this limit is represented by the left-hand side of the rectangle which defines the PI positive events. Moreover, one should realize that the absolute level of the death rate will not really affect our understanding of the cells’ mechanisms.

- On the contrary relative death rates are of great significance. The word “relative” can be given different meanings. In the present paper our main interest is to see how the death rate behaves when the culture moves from the exponential phase to the stationary phase. Does it increase or does it fall? In the framework of our investigation a second possible meaning is to determine whether there is an “infant mortality effect” in the terms of Kelly and Rahn’s paper. It means comparing the death rates of newly divided versus mature cells. We expect a death rate peak for freshly divided cells followed by a steady decline as the size of the cells increases.

5.2 Graph of dead/living

We chose to represent the ratio \( y/x \) rather than the death rate itself for two reasons.

- Whereas the ratio \( y/x \) is read directly on the cytometer, the computation of the death rate relies on a set of assumptions described in the text. It is true that the overall level of \( y/x \) depends upon parameter selection and fine tuning of the cytometer, but the relative variations of \( y/x \) across the 8 measurements performed over a period of five and a half hours are independent of how the parameters of the cytometer have been selected.

- Whereas the absolute level of the death rate may be useful for the purpose of inter-species comparisons, one should realize that anyway such comparisons are necessarily hazardous due to the fact that even for close species such as E. coli and

\footnote{Also to be mentioned is the fact that the number of living cells is affected by the background noise of the cytometer. This is seen very clearly through a test-run in which only PBS+LB (without cells) is introduced in the FC. Then the background noise appears in the form of a set of events at the far left of the domain delimiting the living. This means that the total number of living cells detected by the FC is an overestimate. The purpose of setting an appropriate left-hand side limit for the domain delimiting the living cells is precisely to eliminate these spurious events.}

\footnote{Arguments in favor of a predicted fall were given in a recent separate paper (Di et al. 2021).}
Fig. 2 PI positive events for 8 samples extracted at 45mn time intervals. The scale of the x-axis gives the intensity of PI fluorescence as detected by the cytometer. In spite of the fact that the same procedure was implemented in the successives assays, the distributions show notable fluctuations. Needless to say, these fluctuations affect the estimates of the number of dead cells. It will be the purpose of forthcoming experiments to determine whether these fluctuations can be reduced or whether they are purely random fluctuations.

Fig. 3a,b Optical density and ratio dead/living for 8 samples taken from the culture at 45mn time intervals.
(a) This growth curve serves to define the two regions labelled as “Exponential phase” and “Stationary phase” in Fig. 3b.
(b) This curve gives the ratio dead/living which is denoted \( r = y/x \) in the text. From this ratio it is easy to derive the death rates by using formula (4) given in the text. Note that whereas \( r \) is a number without dimension, for the death rate one must specify the time unit.

**B. subtilis** there are many ingrained differences.

Is there a fall in the death rate \( \mu(t) \) in the course of time as was predicted in Di et al (2021)? The fluctuations are somewhat too large to permit a clear conclusion.
However, it appears that there is no major increase.

**Remark** If the ratio $y/x$ is considered to be approximately steady, formula (3) shows that $\mu(t)$ is proportional to the growth exponent $\alpha$ of the population. As $\alpha$ decreases with $t$ it might seem that $\mu$ will also fall with $t$. Unfortunately, this reasoning is not correct for in solving the differential equation $\alpha$ was supposed constant.

**A note about fluctuations** Fig.3b might suggest a fairly high level of fluctuations. In reality, the absolute level of the fluctuations are of the order of one per 1,000 which means that, compared to similar observations, they are in fact of same order. Here is a comparison.

Using a hemocytometer, Jones et al. (1985, p.78, table 1) determined the proportion of viable spleen cells of mice through a staining method using a mixture of propidium iodide and fluorescein diacetate. In order to assess the accuracy of their method they repeated the measurements 8 times. The coefficient of variation (standard deviation divided by the mean) of the 8 proportions was 40%. For the 8 measurements represented in Fig.3b the coefficient of variation is 51%. In other words, it is only because the proportion that we measure is quite small that the fluctuations depicted in Fig.3b appear to be large.

In addition the graph shows that in order to clearly identify a downward trend, the fluctuations would have to be further reduced by a factor 2 or 3.

6 Conclusion and perspectives

While still open to improvement, our death rate measurements appear more accurate than those of Stewart et al. (2005) and Fontaine et al. (2008). To be fair one should add that the death rate measurement was not the main goal of these papers. The important point in Fig.3b is not so much the absolute level but rather the magnitude of the fluctuations. Although already fairly small, the fluctuations will have to be reduced further in order to see if a trend can be identified. In addition it would be appropriate to add two or three data points in the stationary phase that is to say above the optical density of 1.

Although the cytometric measurements must be handled with great care they appear to be the only promising method for this kind of bio-demographic investigations.
A Appendix A. Death rates in human and bacterial populations

A.1 Global death rates in human populations

For a human population the global death rate of a population $A$ is obtained by first counting the number of deaths in one year (that we denote $\Delta y$) and by dividing this number by the size, $x(t)$, of the population. The ratio $r_d = \Delta y / x(t)$ represents the relative fall of the population.

However, this definition does not give a satisfactory death rate. The reason can be seen by considering neonatal mortality data. Usually they are given for the following successive intervals: (i) less than one day, (ii) from one day to less than one week (iii) from 8 days to less than 4 weeks. Needless to say, $\Delta y$ will be heavily dependent upon the respective time intervals: for (i) it is one day, whereas for (iii) it is 28-7=21 weeks. With a stable number of deaths on successive post natal days the $r_d$ of interval (iii) would artificially appear 21 times larger than for interval (i). If the number of deaths on successive days are not the same (as is indeed the case) this phenomenon will be completely hidden by the change in time intervals. This difficulty can be overcome easily by normalizing $r_d$ by the length of the time interval, thus instead of $r_d$ one should consider: $\mu = r_d / \Delta t$. With this definition identical deaths in successive days will indeed lead to a constant $\mu$. This $\mu$ can be taken as an appropriate definition of the death rate.

The present normalization argument could appear somewhat trivial, but actually in many national data for neonatal death rate statistics, this normalization is not made. Because, $x(t)$ is usually much larger than $\Delta y$, it is often convenient to multiply $\mu$ by 1,000 or equivalently to express $x(t)$ in thousands of individuals.

In addition to the time length of an observation $\Delta t$ just introduced, one must also specify which time unit ($u$) is used. This did not play any role in the previous argument because implicitly it was the day which was selected as time unit. In order to see the role of $u$ consider the following case.

One wishes to measure the death rate between two censuses. In this case, $\Delta t$ is 10 years; if for $u$ we take also 10 years, $\mu$ will give the death rate per 1,000 and per 10 years. Usually, this is not what one wants because one wishes to compare $\mu$ to the annual death rate. To obtain an annual death rate, one should take $u = \text{one year}$. This will give a death rate that is one tenth of the previous one. It represents the average annual death rate over the 10-year period.
A.2 Estimates of the number of life cycles

In our experiment the number of dead *E. coli* cells were measured every 45 mn. However for the sake of simplicity of the present argument we assume that the measurements were performed every 60 mn. Assuming in addition a generation time of 20 mn, there will be 3 doublings. If we assume that they were 10,000 bacteria at initial time $t_0 = 0$, at the end of the 60mn there will be 80,000.

At time $t_1 = 40$mn the population will number 40,000. All these 40,000 cells were born before $t_1$ and all of them had been exposed to the risk of death during their whole life cycle.

If we assume that all cells are synchronized they will all divide at time $t_1$ and the resulting 80,000 cells will be exposed to the risk of dying during their whole life cycle. In this case the number of life cycles would be:

$$N_l = 20,000 + 40,000 + 80,000 = 80,000(1/4 + 1/2 + 1) = 1.75x(t)$$

where $x(t)$ is the end population.

However, in reality the cells are not synchronized. Hence the previous formula should be changed according to the following discussion. It turns out that the formula $N_l = 1.75x(t)$ should be replaced by: $N_l = 1.37x(t)$.

Cells born at $t_2 = 50$mn, will be exposed to the risk of death for only 10mn, that is to say one half of the life cycle; those born after $t_2$ will be exposed to the risk for an even shorter time. Clearly, to continue this argument one needs to know the distribution by age of the third generation.

One can get an idea of the distribution of *E. coli* cells by age at a given moment through the distribution of their lengths. Examples of distributions by size are given in the literature and were found in agreement with our own measurements. They show a substantial peak in young age followed by a slow and fairly steady fall. For the sake of simplicity this kind of distribution will be schematized as having two separate peaks: one at $t = 45$mn corresponding to 3/4 of the generation and a second at $t = 55$mn which corresponds to the remaining 1/4. Those of the first peak will be exposed to the risk of death for 15mn, i.e. 0.75 life cycle, whereas those of the second peak will be exposed for only 5mn, i.e. 0.25 life cycle.

Thus, the total number of life cycles subject to the risk of dying can be written:

$$N_l = 20,000 + 40,000 + 80,000[(3/4) \times 0.75 + (1/4) \times 0.25]$$

which gives:

$$N_l = 80,000 \times (0.25 + 0.5 + 0.62) = 1.37x(t)$$
Now, if one assumes that $n_d$ dead cells have been counted by the cytometer we can apply definition (1) with:

$$x(t) = N_l \quad \Delta y = n_d \quad \Delta t = 1 \text{hour} \quad u = \text{hour}$$

Replacement leads to the following death rate:

$$\mu = 1000 \frac{n_d}{l_c 1.37 x(t)} = 1000 \times 0.82r$$

where $l_c$ denotes the length of a life cycle and $r$ denotes the ratio dead/living given by the cytometer.

Note that the cytometer will also count a number of dead cells whose death occurred before the 45mn-long observation time; but due to the exponential growth this number can be considered negligible compared to the deaths which occurred during the subsequent interval of observation.

If in the previous argument we replace the time interval of 60mn by the one of 45mn which was actually used in our experiment the same argument leads to the following expression for total number of life cycles:

$$N_l = 23,784 + 47,570[(3/4) \times 0.75 + (1/4) \times 0.25] = 1.12 x(t)$$

In writing this expression we have separated the time interval of 45mn into one of 25mn and a second of 20mn. In 25mn the population will reach $2^{25/20} = 23,784$ and all these cells will be able to finish their live cycle. In contrast, the cells born during the last 20mn will not be able to finish their life cycle and will give rise to a number of life cycles that can be estimated by the same argument as above.

This leads to the following expressions for the number of life cycles and for the death rate:

$$N_l = 1.12 x(t) \quad \rightarrow \quad \mu = \frac{1}{l_c 1.12 x} = 1000 \times 1.19r \quad (2)$$

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