POLISH POLAR RESEARCH
vol. 27, no. 1, pp. 63–69, 2006

Differences in the relationship between bacterial count decay and storage time in Antarctic freshwater samples

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Abstract: The mathematical model that described the relationship between cell-count decay and storage time in fixed bacterioplankton samples from three Antarctic lakes of differing trophic status was determined after a one-year experiment. Bacterial density was estimated by epifluorescence microscopy. Cell count data fitted a negative exponential model in all three cases ($p < 0.00001$). However, the slopes of their curves were significantly different ($p < 0.01$), as well as the percentage of bacterial loss after a period of two months. This fact might be related to the limnological characteristics of the water bodies, though the individual genetic variability of their bacterioplankton should not be left aside. Original bacterial numbers in the samples could also be a reason of the differences observed in the pattern of decay in cell counts. Thus, applying a general decay function to any sample and assuming the idea that freshwater bacterioplankton samples can be stored for a two month-period before the bacterial counts decay, can lead to an erroneous estimation of bacterial numbers with direct consequences in ecological investigations.

Key words: Antarctica, bacterioplankton, count decay, storage.

Introduction

Two decades ago, Azam *et al.* (1983) work constituted a springboard for the study of the microbial loop. With burgeoning recognition of the microbial loop as an important pathway for nutrient and carbon flux in aquatic ecosystems, there has been an increasing interest in studying the bacterioplankton from both marine and freshwater ecosystems. The development of fluorescence techniques, particularly epifluorescence microscopy and more recently flow cytometry (Gasol and del Giorgio 2000), has allowed the enumeration of bacterioplankton replacing cell plate counts, which gave at best 10% of the actual numbers (Azam *et al*. op. cit.).

Relevant literature confirms the worldwide importance of the bacterioplankton in the aquatic food webs (Sorokin 1999). In particular for Antarctica, Laybourn-Parry (1997) asserted that the plankton of Antarctic lakes is dominated by the microbial loop, including bacteria, protozoa and phytoplankton. Recently, the dynamics of bacterioplankton was studied in Antarctic lentic ecosystems representing a gradient of saline and trophic conditions (Laybourn-Parry *et al*. 1997; Izaguirre *et al*. 2001; Pearce and Butler 2002; Izaguirre *et al*. 2003). These ecological studies provide evidence of the relevance of bacterioplankton as components of Antarctic freshwater ecosystems.

Several factors can lead to the storage of fixed bacterioplankton samples. For example, due to the intense sampling frequency during Antarctic summer and whole year surveys, and the sometimes impracticability of counting the bacteria present in the water samples in the Antarctic stations, investigators often take large numbers of preserved samples back to their laboratories. Moreover, as there is a significant variability introduced in cell counts if more than one investigator performs the countings (Kirchman *et al*. 1982; Kepner and Pratt 1994) a consequent delay in the analysis of the samples may occur when only one investigator is involved in the counting. Turley (1993) has already discussed the bacterial cell loss due to sample storage of preserved seawater samples, concluding that the counts from stored samples for more than a two-month period may underestimate bacterial numbers and their importance in aquatic ecosystems.

The aim of this study was to find a mathematical relationship between cell count decay and storage time in glutaraldehyde-fixed and DAPI-stained bacterioplankton samples from Antarctic freshwater ecosystems of differing trophic status at Hope Bay (Antarctic Peninsula), and to evaluate the bacterial count decay in them.

Methods

Field samples from three freshwater water bodies at Hope Bay (63°23'S, 57°W), Maritime Antarctica, were used in the bacterial count decay long-term study. Three replicate water samples were collected at subsurface level in 1 liter PVC bottles (Kirchman *et al.* 1982) from water bodies with differing trophic status: Pingüi pond (hypertrophic), Lake Boeckella (mesotrophic) and Lake Chico (oligotrophic) (Izaguirre *et al.* 1998). Samples were immediately fixed with 2% ice-cold glutaraldehyde and preserved in the dark at 4°C until they were processed. Twelve aliquots (2 ml) from each of these replicates were DAPI-stained, filtered and mounted on slides for bacterial-cell count following Izaguirre *et al.* (2001). They were stored at –20°C in the dark until their enumeration. Due to the impracticability of counting the samples in the Antarctic laboratory, the initial density (N_i) was the one obtained from the first observation performed within the first two-month period after the sample was collected. In this sense, and as it was proposed

by Turley (1993), it was assumed that no decay in bacterial counts took place during that period. The rest of the samples were observed during a year after the first observation. Bacterioplankton cells were counted by a single person to avoid variability, using a Zeiss Axioplan epifluorescence microscope (×1000) with UV light (excitation filter BP 365 nm, dichromatic beam splitter FT 395 nm, barrier filter LP 397 nm). At least 400 cells for each replicate were counted, corresponding to a minimum of 20 fields of view (Kepner and Pratt 1994).

Data analysis

Bacterial cell count data from each of the three water bodies was adjusted to different mathematical models to determine which function the data fitted best. For each model, a simple regression was performed to test the null hypothesis H_0: $\beta = 0$ (β = slope of the curve). The model which presented the higher r (Pearson's correlation coefficient) and the smallest RMS (residual mean square) was chosen as the one that best represented the bacterial cell count decay.

In order to test the significances of the differences among the slopes of the cell-count decay function of the three water bodies, an analysis of covariance (ANCOVA) was employed using ln-transformed cell counts. The null hypothesis (H_0: $\beta_P = \beta_B = \beta_{Ch}$) that represents the equality of bacterial-cell count decay in Pingüi, Boeckella and Chico water bodies was tested. A *post hoc* multiple comparison procedure (Tukey test) was employed to assess for differences between each pair of β values (Zar 1996).

Results

Initial bacterial density (N_i) differed among the lakes, obtaining the greatest values in Lake Boeckella samples ($1.44 \times 10^6 \pm 2.48 \times 10^4$ bact. ml^{-1}) followed by Lake Chico ($1.59 \times 10^5 \pm 1.24 \times 10^4$ bact. ml^{-1}) and Pingüi Pond ($1.56 \times 10^5 \pm 5.79 \times 10^4$ bact. ml^{-1}) (Fig. 1).

In all three water bodies, cell count data adjusted an exponential decay model of the form:

$$N_t = N_0 e^{\beta t} \quad (1)$$

where N_t is the number of bacterial cells counted at time t, t is the time of sample storage in days, N_0 is the number of cells at time zero and β is the value of the slope (Fig. 1a, b and c). The ANOVA revealed a good fit to this mathematical function ($p < 0.00001$) in the three studied cases. The ANCOVA showed significant differences among the estimated β values of all three curves ($p < 0.001$): $\beta_{Chico} = -0.014$; $\beta_{Boeckella} = -0.012$; $\beta_{Pingüi} = -0.017$. The Tukey's test *post hoc* analysis

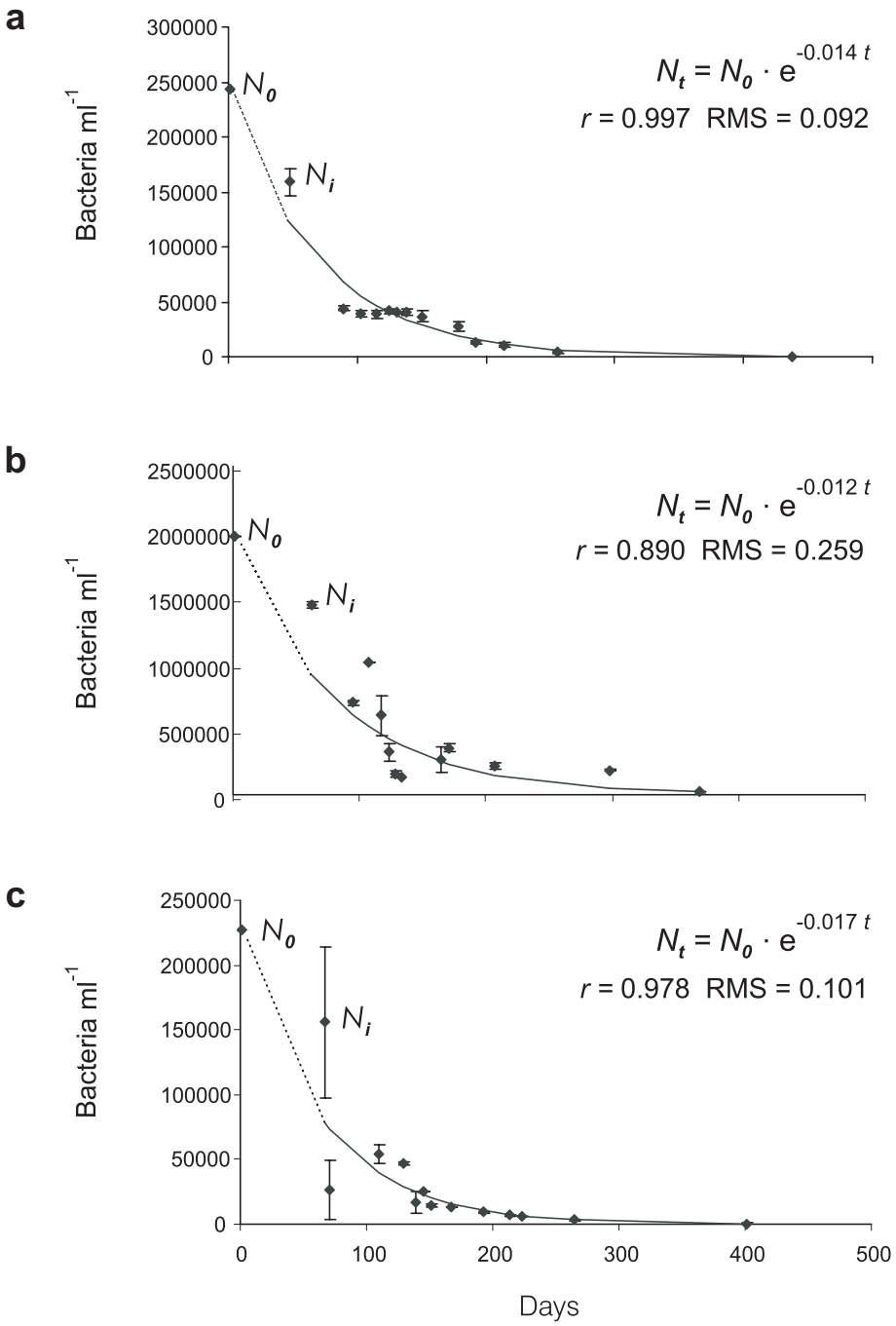

Fig. 1. Bacterial cell number vs. time for the three studied water bodies: Lake Chico (a), Lake Boeckella (b) and Pingüi Pond (c). N_i – density estimated in the first observation performed within the first two-month period, N_0 – density calculated using the fit curve, t – time of sample storage (days), r = Pearson's correlation coefficient, RMS = residual mean square.

confirmed this result and showed significant differences between each pair of slopes ($p < 0.01$).

The original densities at time zero (N_0) were calculated using the fit-curve for each water body, obtaining marked differences with those resulting from the first observation performed within the two month period (N_i). The calculated original density for Lake Boeckella resulted in 2.56×10^6 bact. ml^{-1}, 4.64×10^5 bact. ml^{-1} for Lake Chico and 7.27×10^5 bact. $ml^{-1}$for Pingüi pond.

The percentage of loss in bacterial numbers was calculated at each observation date for each water body. When the percentage of loss was calculated considering the initial density as N_i, it was clearly observed that the decay was very strong at the second observation date (about 3 months after the collection of the sample), accounting more than 50% in Boeckella Lake, and reaching about 75% both in Chico Lake and Pingüi pond. However, if the percentage of loss in bacterial numbers was calculated considering as the original density the one estimated with the fit curve (N_0), and assuming that the pattern of decay between the day of collection of the sample and the first observation followed the exponential curve, the analyses revealed the greatest decay in Lake Chico samples, followed by Pingüi pond and Lake Boeckella. In this sense, a decay that could represent a range from 30 up to almost 65% loss was calculated. Under this scenario, bacterial numbers strongly dropped within the two months in the three water bodies, and the decay was even more marked after a three month period.

Discussion

The long term experiments revealed that an exponential decay model is the mathematical function that relates the effect of storage time on bacterial cell counts in three Antarctic lakes of differing trophic status. The same pattern was observed in the three studied water bodies independently of their limnological characteristics. This result is consistent with previous reports for Antarctic systems (Tackacs and Priscu 1998), and brings new insight on the fact that the curves obtained for each water body differed in the absolute value of their slopes. This suggests that a negative exponential function could mathematically describe the influence of sample storage time on bacterial cell counts, and the slope of the curve differs. The differences observed among the water bodies in the slopes of their decay curves might be related to the limnological characteristics of the freshwater ecosystems (*i.e.* organic matter, suspended solids, *etc.*), but the individual genetic variability of the bacterioplankton in the water body should not be put aside. In this sense, it would be interesting to study the decay according to the bacterial types by means of molecular techniques.

Turley (1993) proposed that seawater samples could be stored for up to 70 days with no cell decrease. This does not seem to be reliable for the studied fresh-

water lakes. The results revealed that the samples from the three water bodies would have been subject to a sharp bacterial count-decay within the first two-months period, if N_0 was considered. After the two month period the density strongly decreased in all cases. Thus, in relation to the 70 days period proposed by Turley (*op. cit.*) as acceptable to prevent the bacterial count decay in seawater samples, it is probable that in freshwater samples the pattern of decay differs. Again, the chemistry of the water and the genetic composition of bacterioplankton might differ if seawater and freshwater samples are considered, an thus the behavior of decay of their bacterial counts should not be supposed to be the same. However, it must be pointed out that as N_0 is an estimated value this decay should be further evaluated in the first two month period after obtaining the freshwater samples.

As regards the initial bacterial densities, the difference registered among the lakes might account for the variation of the slopes. The differences in the original bacterial numbers of the water bodies might affect the decay rate. Even though this study does not present sufficient data to make a concluding remark since only three water bodies were studied, it is observed that the greatest decay was recorded in the samples that originally contained less bacteria and the smallest in those with more bacterioplankton.

It can be concluded that applying a general decay function to any freshwater sample can lead to an erroneous estimation of the bacterial density. In this sense, the importance of the bacterioplankton in the microbial loop can be misinterpreted with direct consequences in ecological investigations. Thus, it must be stressed that though equation (1) potentially applies for different water bodies, β is to be calculated for each one of them. As well, it is proposed that without a calculated equation for correcting the bacterial counts for a certain freshwater ecosystem, a two-month period might be too long to store freshwater samples with a significant underestimation of the original bacterioplankton densities. However, as the mentioned period was not deeply studied in the present survey, it would be interesting to make a similar study performing more frequent observations within the first two-month period so as to confirm this idea.

Acknowledgements. — Thanks are due to Dr I. Izaguirre and Lic. P. Almada for their assistance in the field work and the members of *Esperanza* Station (CAV 2001) for the logistic support. I am very grateful to Lic. R.J. Lombardo for his statistical support and Dr I. Izaguirre, Dr P. Pizarro, Dr J. O'Farrell and Dr F. Unrein for their useful comments. The work was supported by ANPCYT (BID 802/OC-AR-PICT 04540) and the Instituto Antártico Argentino (DNA).

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Received 1 June 2005
Accepted 20 December 2005