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Influence of light and natural microbiota of the Butrón river on *E. coli* survival

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Abstract. The survival of an *E. coli* strain in water samples from the Butrón river has been studied. The input of *E. coli* cells in the aquatic system breaks down the established balance among the components of the natural microbiota: *E. coli* becomes the object of the active protozoal predation whereas the autochtonous heterotrophic community become alternative preys. As a result of this new situation, the natural microbiota increases but returns to the initial values once the *E. coli* cells have been removed from the system. The effect of the temperature of incubation on the survival is exerted through the effect of this parameter on the predatory activity of the protozoa. Light has a lethal and direct action on the *E. coli* cells, the effect of this parameter is even superior to that of predation.

INTRODUCTION

The survival of *E. coli* in the aquatic systems is a complex phenomenon regulated by a great variety of factors. Various physical, chemical and biological factors such as temperature (Kittrell & Furfari 1963; McFeters & Stuart 1972; Davenport et al. 1976; Verstraete & Voets 1976; Anderson et al. 1983; Gameson 1984), competition (Jannasch 1968; Lechevalier & McFeters 1985), toxicity (Sieburth & Pratt 1962; Saz et al. 1963; Cabridence & Lepailleur 1969), predation (Enzinger & Cooper 1976; McCambridge & McMeekin 1979, 1980, 1981) and solar radiation (Jagger 1975; Grigsby & Calkins 1979; Kapuscinski & Mitchell 1981; Gameson 1984) have been suggested as playing a role in bacterial survival.

Normally, the effect of a specific parameter on the survival of *E. coli* is studied in the laboratory without considering that the variations of the parameter can cause changes in the behaviour of the natural microbiota which will have an influence on survival. It is reasonable to suppose that the input of sewage, containing large amounts of faecal bacteria, into the aquatic system will induce some variations in the relations established among the components of the natural microbiota.

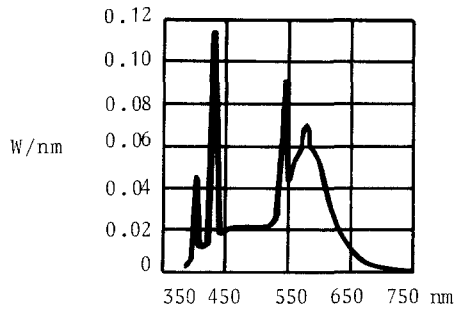

Fig 1. Luminous spectrum of the lamps employed.

In this paper, three are studied i.e. the survival of *E. coli* in relation to the response of the natural microbiota to the input of *E. coli* cells; the effect of the temperature on the natural microbiota with reference to the survival of *E. coli*; and finally the effect produced by light on the survival of *E. coli*.

MATERIALS AND METHODS

The study was carried out with samples of the river Butrón (Biscay, Spain). All samples were collected from the surface using appropriate instruments.

An *E. coli* strain isolated from a river water sample expressed as IMViC (++−−) and Eijkman test + was used. Suspensions of 10^{10}, 10^{8} and 10^{6} *E. coli* cells per ml obtained from a 18 h culture in lactose broth were used as inoculum. The cells were harvested by centrifugation and washed three times with saline solution (0.9%, w/v). All the experiments were carried out in flasks of 250 ml with 100 ml of sample (river water) and 1 ml of inoculum. The experiments with the three inocula were done at 20 °C in darkness. The influence of the temperature was studied with inocula of 10^{10} *E. coli* cells per ml at 28, 20 and 10 °C in darkness. To determine the effect of light, subsamples with inocula of 10^{10} *E. coli* cells per ml were incubated at 28 °C and illuminated by 8 Sylvania CW-ST 133 lamps of 18W (10,000 lumens). The luminous spectrum of this type of lamp is shown in Fig. 1. The experiments were done in an incubator Psycrotherm (New Brunswick) shaking at 200 r.p.m. The number of viable *E. coli* cells was determinated daily on EMB Agar. T_{90} was employed as the representative parameter of the survival. This parameter was derived from the plots of the *E. coli* survival curves.

The community of heterotrophic bacteria was quantified daily by the viable count method using plates of Nutrient Agar (NA) incubated during 48h at 20 °C. The procedure employed to count protozoa was a modification of Singh's (1955) ring method described by Habte & Alexander (1978). Immediately after pouring melted water agar (1.5%) into the Petri dishes, 5 sterile glass rings were placed

in each plate. When the agar was well set, 0.1 ml of a suspension containing *E. coli* cells was added to each ring. 0.5 ml of sample was added as inoculum, and the plates were incubated 4 days at 20 °C. Protozoa were counted by recording the number of rings with or without protozoa as observed microscopically and then estimating the abundance of protozoa by the MPN method.

The quantification of bacterial predators (P.F.U.) was calculated, with slight modifications, following the technique of Stolp & Starr (1963). A suspension of agar (1.2%) in Petri dishes was used as a base and it was covered with 4 ml of another suspension of agar (0.6%) which also contained 0.5 ml of the sample and 0.5 ml of *E. coli* suspension. This was then incubated at 20 °C for 20 days. For the quantification of the lythic population, cycloheximide (final concentration 500 mg/l) was added to the second layer.

To prevent the growth of protozoa, 2 ml of cycloheximide (final concentration 500 mg/l) was added to the flasks with the corresponding subsamples.

RESULTS

All the experiments presented below were carried out 4 times. Given that the results obtained were quite similar and given that the subsequent discussion deals with the development, over a period of time, of the different microbial communities, only one experiment representative of those carried out, for each case is presented here.

The evolution of the natural river microbiota i.e. the heterotrophic bacteria, the protozoa and the lythic population, is shown in Fig. 2a. The heterotrophic bacteria and the protozoa present some oscillations. The lythic population presents an increase of two orders during the experiment and a clear relation between the evolution of this population and the two mentioned above is not observed. The elimination of the eucaryota by the addition of cycloheximide (Fig. 2b), causes changes in the evolution of the community of heterotrophic bacteria manifested in a general increase of this community. However, changes in the evolution of the lythic population, relative to those observed in Fig. 2a, are not observed.

The evolution of the natural microbiota in the presence of three different initial densities of *E. coli* 10^8, 10^6 and 10^4 cells per ml is shown in Fig. 3. When *E. coli* is added to sterile subsamples at an initial density of 10^8 and 10^6 cells per ml, the viable counts in these sterile subsamples do not change during the incubation time (Fig. 3a and 3b). However, in the case of 10^4 cells per ml (Fig. 3c), the number of viable cells increases up to 578,000 cells per ml (72 h), maintaining this density to the end of the experiment (120 h). This *E. coli* behaviour is repeated in all the experiments carried out with this initial density (10^4 cells per ml). It therefore appears that the high concentration of particulate organic

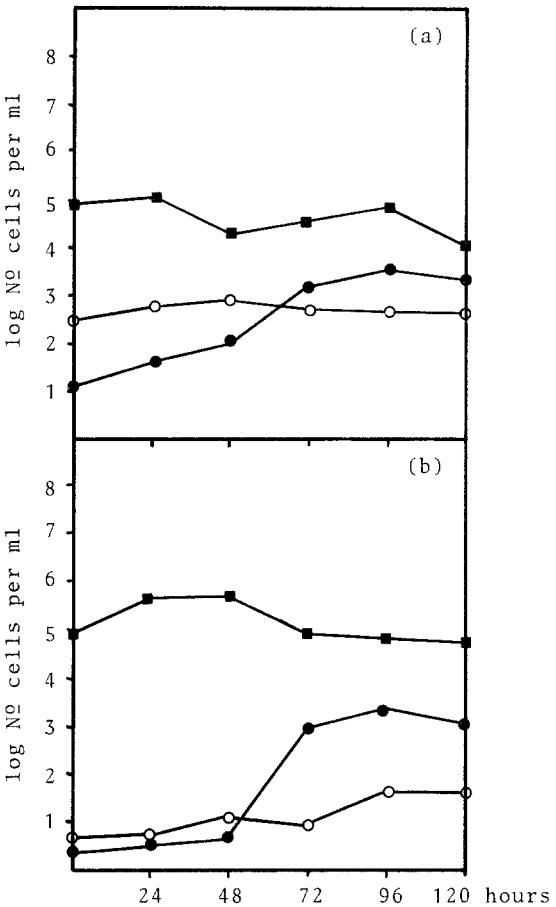

Fig. 2. Evolution of the river microbiota (a) without the addition of cycloheximide and (b) with the addition of cycloheximide.
(■) heterotrophic bacteria, (○) protozoa , and (●) lythic population.

carbon present (6,097 mgC/m^3 in this sample) supports the growth of the *E. coli* population up to that levels, but not higher.

The evolution of *E. coli* in the non sterile normal subsamples is different. When the initial density is about 10^8 cells per ml (Fig. 3a) a typical die-off curve can be observed with a lag and an exponential death phase. The T_{90} of this experiment is 32.5 h. The community of heterotrophic bacteria undergoes, simultaneously with the *E. coli* lag and exponential death phases, an increase from 10^4 to 10^6 and a decrease from 10^6 to 10^4 respectively. From the 48 hours of incubation to the end of the experiment the community of heterotrophic bacteria increases slowly. The protozoal population increases up to 48 hours when its density is maximum (73,000 cells per ml). At this moment the exponential death phase of *E. coli* and the decrease of the community of heterotrophic bacteria

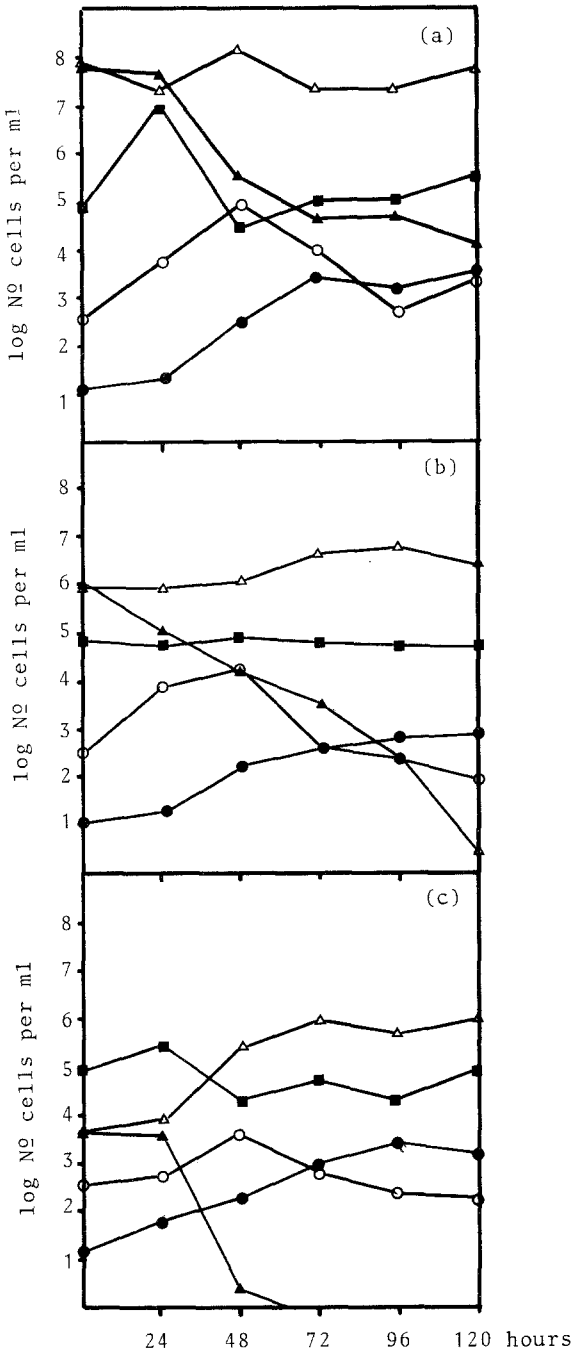

Fig. 3. Evolution of the natural microbiota in the presence of different initial densities of *E. coli*: (a) 10^8 cells per ml, (b) 10^6 cells per ml, and (c) 10^4 cells per ml.
Sterilized subsample: (△) *E. coli.*
Normal subsample: (▲) *E. coli*, (■) heterotrophic bacteria, (○) protozoa, and (●) lythic population.

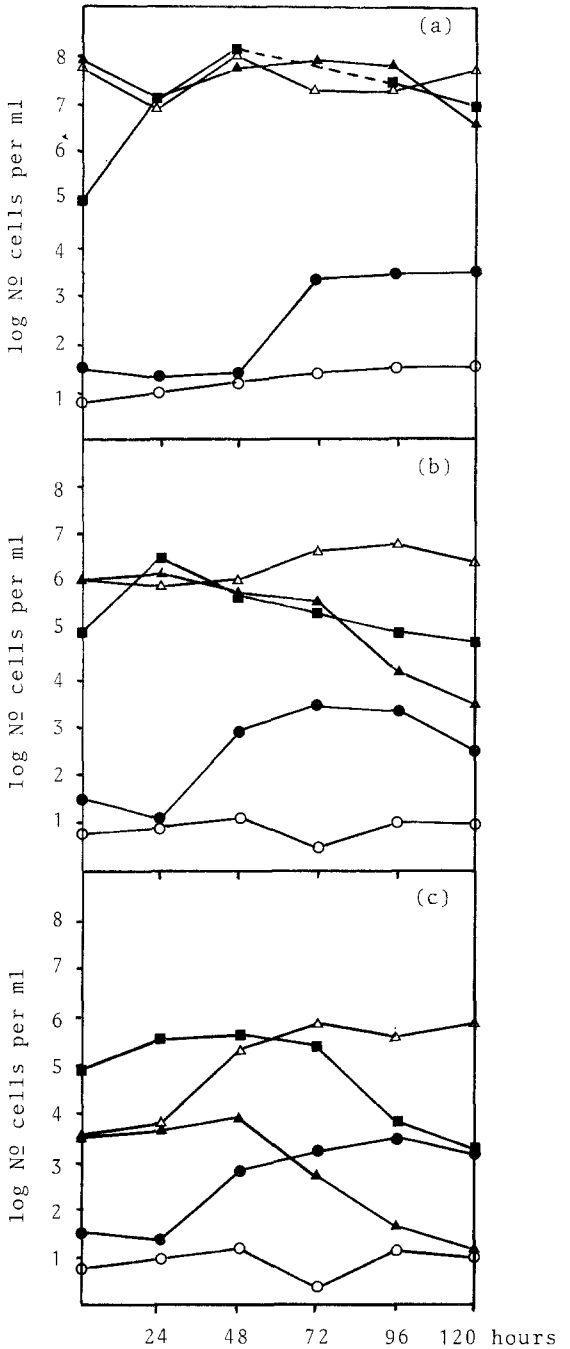

Fig. 4. Evolution of the natural microbiota, with the addition of cycloheximide, in the presence of different initial densities of *E. coli*: (a) 10^8 cells per ml, (b) 10^6 cells per ml, and (c) 10^4 cells per ml.

Sterilized subsample: (△) *E. coli.*

Normal subsample: (▲) *E. coli*, (■) heterotrophic bacteria, (○) protozoa, and (●) lythic population.

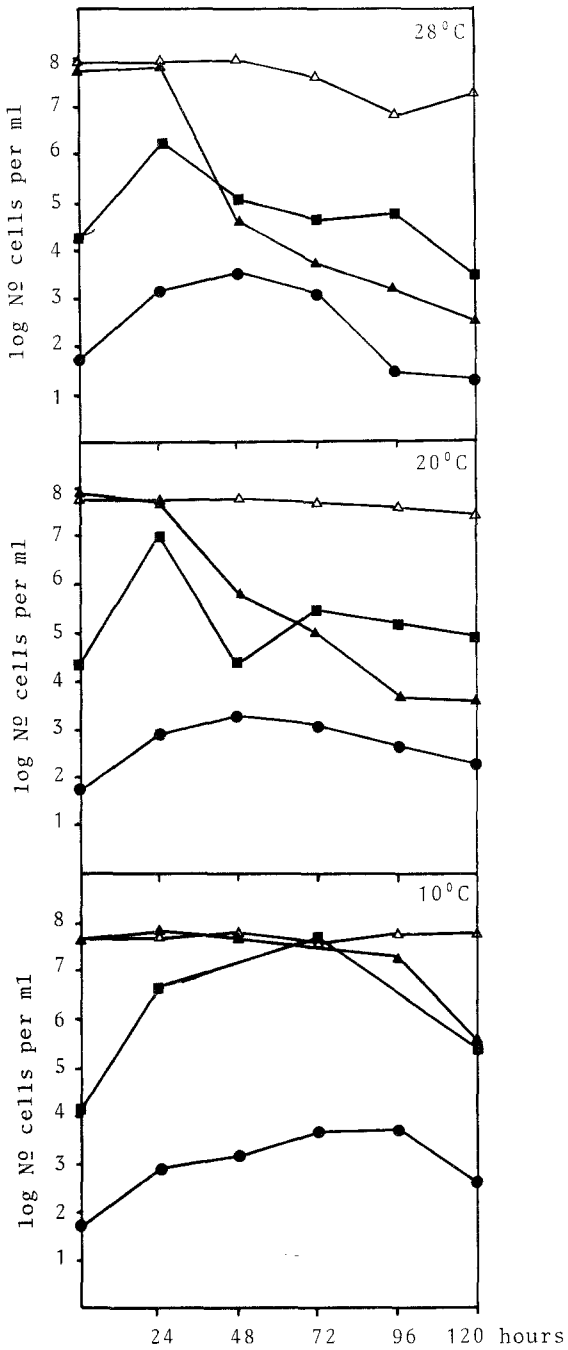

Fig. 5. Evolution of *E. coli* and natural microbiota at 28, 20 and 10°C.
Sterilized subsample: (△) *E. coli.*
Normal subsample: (▲) *E. coli,* (■) heterotrophic bacteria, and (●) P.F.U.

end. Subsequently, the number of protozoa decreases significantly. Finally, the lythic population presents at 72 h a maximum of 2,540 cells per ml.

When the initial density of *E. coli* is 10^6 cells per ml (Fig. 3b) a continued decrease during the experiment is observed. The T_{90} value in this case is 23 h. Major oscillations of the heterotrophic community are not observed, whereas the protozoal population shows a similar evolution as in the previous case, i.e. an increase up to 48 h and, subsequently, a decrease. The lythic population exhibits the same evolution as observed with the higher inocula, although in this case the maximum cellular density is reached after 96 h of incubation.

When the initial density of *E. coli* is 10^4 cells per ml (Fig. 3c) a 24 hour lag phase is followed by an exponential death phase. The T_{90} value is 32 h. The heterotrophic community and protozoal population present a typical evolution predator-prey in the first 72 hours; from this moment to the end of the experiment the protozoal population decreases whereas the lythic population reaches its maximum density.

The evolution of the *E. coli* and the natural microbiota in the absence of protozoa (subsamples with cycloheximide) is shown in Fig. 4. When comparing these results with those obtained in the subsamples without cycloheximide (Fig. 3), three differences are observed. First, the survival of *E. coli* increases considerably and T_{90} values of 112, 82 and 77 hours in the subsamples without protozoa and 32.5, 23 and 32 hours in the subsamples with protozoa are obtained. Second, the heterotrophs obtain higher populations in the subsamples without protozoa. Third, the lythic population is lower at 24 h in the subsamples without protozoa. These three differences can be observed for the three initial densities of *E. coli* (10^8, 10^6 and 10^4 cells per ml).

To study the effect of the temperature on the survival of *E. coli,* three experiments with water samples were done at 28, 20 and 10 °C in darkness and with an initial density of 10^8 cells per ml (Fig. 5). No significant differences of evolution in the sterile subsamples at these three temperatures were observed in the period of incubation studied. In the non sterile subsamples, the survival increases when the temperature of incubation decreases, registering T_{90} values of 31.4, 34 and 105 hours at 28, 20 and 10 °C respectively. The heterotrophic and predator communities have, at 28 and 20 °C a similar evolution to that previously described in Fig. 3a. However, at 10 °C the general evolution of the populations is very different, the *E. coli* lag phase is prolonged another 72 hours where the density for the heterotrophic community reaches its maximum ($25 \cdot 10^6$ cells per ml). The predators reach their maximum at 96 hours and at this moment, the *E. coli* population and the heterotrophic community start to decrease.

To study the effect of light on the survival of *E. coli,* four experiments with a water sample – with and without light – were done at 28 °C, and with an initial density of 10^8 cells per ml (Fig. 6). The T_{90} of the illuminated subsamples, sterile and non sterile, are 25 and 8 hours respectively, whereas the T_{90} of the non

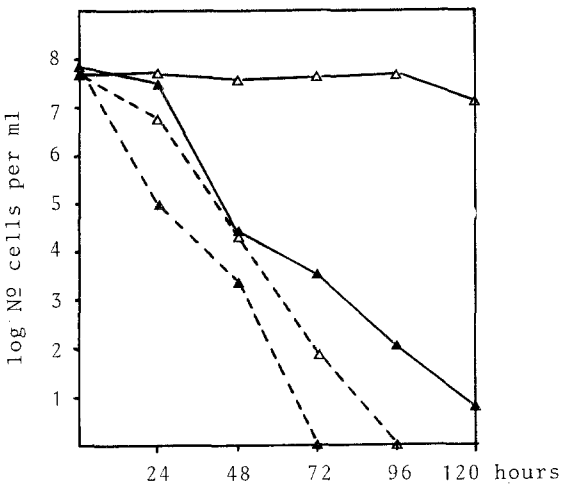

Fig. 6. Effect of light on *E. coli* survival.
Sterilized subsample: (△---△) illuminated and (△——△) non illuminated.
Normal subsample: (▲---▲) illuminated and (▲——▲) non illuminated.

sterile subsample in darkness is 28.5 hours. It can be observed that the combined effect of light and the natural microbiota drastically reduces the *E. coli* survival.

DISCUSSION

The death of *E. coli* is mainly caused by predation phenomena as can be deduced from the differences in the evolution of *E. coli* death plots (Fig. 3) in which the natural microbiota is present or absent.

In all experiments, a maximum in the evolution of protozoa, coinciding with the end of the exponential phase of *E. coli* death, is observed. When the eucaryotes are removed by the addition of cycloheximide to the subsamples (Fig. 4) the survival of *E. coli* increases greatly. In this manner, T_{90} values of the subsamples with protozoa (32.5, 23 and 37 hours) are much lower than those without protozoa (112, 82 and 77 hours). So, we can assure that the predation exerted by protozoa is one of the more important causes of *E. coli* death.

These deductions agree with those obtained by Enzinger & Cooper (1976) and McCambridge & McMeekin (1980) in an estuary. These authors consider, moreover, that the contribution to the predation exerted by *Bdellovibrio* and other lythic bacteria is negligible as opposed to the predatory activity of the protozoa. In this respect, in the absence of protozoa, we have observed that the influence of the lythic population on *E. coli* death is minimal and is only exercised after 72–96 hours of incubation (Fig. 4).

Fig. 2a shows that the heterotrophic bacteria and the protozoa coexist with a determined dynamic predator-prey. When *E. coli* cells are introduced into the system (Fig. 3a) the balance is disturbed and the predator population grows at the expense of this new prey. This change of prey is favoured by the fact that the new prey has a nutritional value for the eucaryota predator which is higher than the wild prey (Taylor 1978). Furthermore, the ciliates – which are main constituents of the protozoal population in our experiments – can select their prey in terms of its nutritional value (Dive 1973).

Danso & Alexander (1975) have reported that when protozoa coexist with various preys, the active predation is exercised over the most abundant and the others remain as alternative preys. In our case, the heterotrophic bacterial community of the river is the usual prey of the protozoa and when *E. coli* cells are introduced into the system a qualitative change is produced making *E. coli* the object of the active predation and the heterotrophic community the alternative prey.

Between 0 and 24 hours of incubation the viable count of *E. coli* remains constant whereas the heterotrophic community and the protozoa increase. The increase of the protozoa may be due to the contribution of non-viable cells incorporated with the inoculum of *E. coli*. This should explain the constancy of the viable *E. coli* count, as well as the increase of the heterotrophic community since it has became an alternative prey. As can be observed in Fig. 3, this phenomenon occurs with all the inocula essayed and the intensity is related to the number of *E. coli* cells introduced into the system.

It is known that temperature has an influence upon survival (Kittrell & Furfari 1963; Gameson 1984). However, our experiments reveal that this influence does not exist when the microbial community of the river is absent (sterile subsamples). In this situation, differences in the evolution of *E. coli* death plots at the three temperatures essayed (28, 20 and 10 °C) are not observed (Fig. 5). On the other hand, when the natural populations of the river are present (non sterile subsamples) *E. coli* survival increases inversely to the incubation temperature (Fig. 5).

It could be assumed that the T_{90} values of the three temperatures tested, would change proportionally to the gradient of temperature. However, this proportionality does not exist: T_{90} values at 28 and 20 °C (31.5 h and 34 h) are significantly smaller than those at 10 °C (105 h). Fig. 5 shows that the evolutions of microbial populations at 28 and 20 °C are very different than those at 10 °C. These results point out that the influence of temperature on survival is exerted throughout the natural microbiota of the river. It must be considered that the temperature is a physical parameter with a decisive influence on the microbial activity. In this manner, temperatures of 28 and 20 °C are in accordance with the high activity levels whereas at 10 °C the activity level of the microorganisms is very low (Costerton & Colwell 1979).

The influence of light on the *E. coli* survival is shown in Fig. 6. The differences between the death plots corresponding to the sterile subsamples with and without light indicate that this parameter has a great lethal action on the *E. coli* cells. When the natural microbiota is present (non sterile subsamples) the predatory effect is combined with the lethal action of the light and consequently the survival is very low – T_{90} 8 h (Fig. 6). It must be emphasized that the lethal action of light is similar to the predatory effect of natural microbiota. The T_{90} value of the sterile subsample cultured with light (25 h) is very similar to the T_{90} of the non sterile subsample cultured without light (28.5 h).

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