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Role of Hydrogen Peroxide in Loss of Culturability Mediated by Visible Light in *Escherichia coli* in a Freshwater Ecosystem

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A study was made of the mechanisms by which visible light produces cell dormancy in *Escherichia coli*, resulting in loss of culturability. Visible light may act directly on the cells or generate photoproducts with a negative effect on the cells. In nonilluminated microcosms the addition of increasing concentrations of hydrogen peroxide, one of the photoproducts formed in natural aquatic systems, gave rise to the formation of nonculturable cells and injured culturable cells, and this negative effect depended on the concentration of peroxide. On the other hand, in illuminated microcosms the addition of compounds which eliminate hydrogen peroxide (i.e., catalase, sodium pyruvate, and thioglycolate) had a protective effect on the *E. coli* cells, as the CFU counts on minimal medium and on recuperation medium were significantly higher ($P < 0.05$) than those detected in the absence of these compounds. Furthermore, when hydrogen peroxide was eliminated, the CFU counts on recuperation medium did not fall significantly, indicating that nonculturable cells did not form. These results rule out the direct effect of visible light on the cells and show that hydrogen peroxide, generated photochemically, may be the cause of the loss of culturability of *E. coli* in illuminated systems.

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The survival of *Escherichia coli* in aquatic systems is controlled by the biotic and abiotic factors of the systems. Among the biotic factors which affect the survival of *E. coli*, the predation by protozoa, mainly nanoflagellates and ciliates, is especially noteworthy (1, 3, 6, 15, 16, 24). Other biotic factors, such as competition (21, 22) and lysis produced by bacteriophages and lytic bacteria (11, 16, 19, 26), are of less quantitative importance. With regard to the effect of abiotic factors, visible light is one of the factors which contribute most efficiently to the decrease in the number of *E. coli* CFU in natural systems (2–5, 10, 12).

Several authors (9, 30) indicate that the negative effect of light is mediated by a photosensitizer and the reactions almost always involve the participation of molecular oxygen. These photosensitized reactions may yield the production of highly reactive oxygen species: singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals.

Recently, the authors (2, 4, 5) have reported that visible light causes a dormancy process in *E. coli* cells. One of its consequences is the loss of culturability of these cells, as well as inhibition of the incorporation of glucose and of biosynthetic processes and a drop in the number of metabolically active cells.

This dormancy process and the loss of culturability which it involves make the detection of cells exposed to visible light impossible by means of traditional counting methods on plates. These facts have serious repercussions for public health because of the use which is normally given to *E. coli* as an indicator of fecal contamination in water. Moreover, several authors (18, 28, 29) have found that these nonculturable cells can maintain their infective capacity and pathogenic potential.

This means that methodologies must be revised to allow the recuperation of these nonculturable cells in order to detect them on selective or minimum culture media on which only the indicator microorganism grows. However, to attain this ambitious goal it is first absolutely essential to determine some conceptual aspects concerning the way light acts, and this was the aim of this study. In this regard, we shall study whether the cell dormancy process is produced as a consequence of the direct action of the light on the cells or if it is produced as a consequence of the effect of the products generated photochemically upon the cells.

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MATERIALS AND METHODS

This study was carried out with water samples from the Butrón river (Vizcaya, Spain). All samples were collected from the surface in sterile glass bottles. The total organic carbon (TOC) concentration in the samples was measured with a Maihack Defor infrared analyzer (model Tocor 1; Westinghouse, Hamburg, Germany), and its mean value was 15.5 mg of C liter^{-1}.

An *E. coli* strain isolated on MacConkey agar plates from a river water sample and confirmed by the API 20E System (Bio-Merieux) was used throughout this study.

Inoculum preparation. *E. coli* was grown in nutrient broth at 28°C for 8 h. Cells from the exponential phase were harvested by centrifugation (3,000 × *g* for 15 min) and washed three times with sterile saline solution (0.9% [wt/vol]). The pellet was suspended in saline solution, inoculated in fresh nutrient broth, and incubated at 28°C for 18 h. The cells were harvested at stationary phase as described above. This final suspension was inoculated in the water samples at a final density of approximately 10^8 cells ml^{-1}.

Experimental design. All experiments with illuminated microcosms were carried out in 250-ml Pyrex glass flasks containing 100 ml of sterile subsamples obtained by filtering river water through 0.22-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). An orbital incubator with an illumination system consisting of seven Sylvania F30

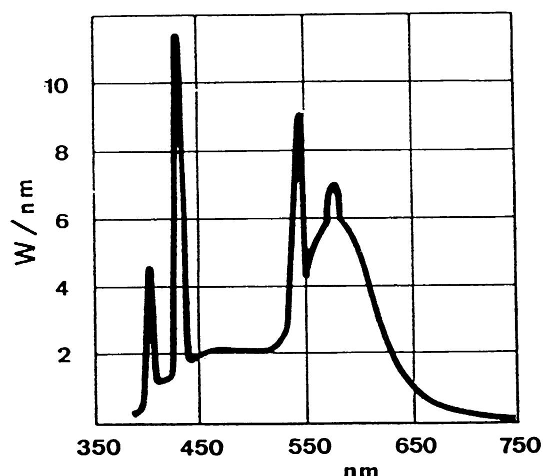

FIG. 1. Luminous spectrum of the lamps employed.

W/T8/D lamps was used. The luminous spectrum of these lamps is shown in Fig. 1. In all experiments, we prepared nonilluminated control flasks covered with aluminum foil. Incubation was done at 20°C with shaking at 180 rpm.

Experiments were made with subsamples of illuminated water to which catalase (11 U ml^{-1}, final concentration), sodium pyruvate (1 mg ml^{-1}, final concentration), or sodium thioglycolate (0.05%, final concentration) was added. Catalase and pyruvate were added at 0 and 24 h of incubation, and thioglycolate was added every 24 h, from 0 to 72 h of incubation.

Experiments in which hydrogen peroxide was added to the sterile nonilluminated subsamples were made in 65-ml glass bottles (acid cleaned) containing 50 ml of sterile subsamples obtained as described above. Increasing final concentrations of hydrogen peroxide in the river water subsamples (0.001, 0.005, 0.01, 0.05, 0.1, and 0.2%) were obtained from a 3% commercial solution. The treated subsamples and the control (without hydrogen peroxide) were incubated for 15 min at 20°C. After this period of contact, the hydrogen peroxide was neutralized with a sterile solution of catalase (11 U ml^{-1}, final concentration). All the additional solutions added were sterilized beforehand by filtration (0.22-μm-pore-size membrane filters).

Bacterial counts. CFU of *E. coli* were enumerated on a recuperation medium, lactose nutrient agar (LNA) (23), and on a minimal medium, minimal glucose agar (MGA) (23). The MGA with catalase was processed by adding catalase to the medium in a final concentration of 275 U per plate. The MGA medium supplemented with sodium pyruvate was processed by adding 1 g $liter^{-1}$ to the medium.

The total number of cells was estimated by the acridine orange direct count (AODC) method of Hagström et al. (20) in subsamples preserved in 2% (final concentration) formaldehyde.

Statistical analysis. Statistical tests were done with the StatView program (Apple Computer). All counts reported are the means of at least three determinations, and the coefficients of variation between replicate experiments were less than 12%. The differences between the means were detected by a one-way analysis of variance. Probabilities less than or equal to 0.05 were considered significant.

RESULTS

In nonilluminated systems, the total number of cells (AODC), as well as the CFU counts determined on LNA and on MGA, remained constant throughout the incubation period (data not shown). On the other hand, in illuminated systems (Fig. 2A), although the total number of cells remained constant, the number of culturable cells determined as CFU both on the LNA and on the MGA gradually decreased from the beginning to the end of the experiment. After 48 h of incubation, statistically significant differences ($P < 0.05$) were obtained with respect to the initial number of CFU determined on both media. Furthermore, as the time of exposure to visible light was increased, significant differences were detected ($P < 0.05$) between the counts obtained in both culture media. These reductions in the CFU counts on both of the culture media used indicated that light produced an increase throughout the exposition time in the number of nonculturable cells (AODC minus CFU counts on LNA) as well as in the number of injured culturable cells (CFU counts on LNA minus CFU counts on MGA).

The effect of hydrogen peroxide on *E. coli* cells was assayed in nonilluminated systems (Fig. 3). After 15 min of contact with the lowest concentration of peroxide used (0.001%) a slight drop was produced in the CFU counts on both culture media. With 0.01% peroxide, the differences ($P < 0.05$) obtained between the CFU counts on the LNA and those on the MGA indicated the appearance of a fraction of injured culturable cells. In a similar way, with 0.05% hydrogen peroxide, statistically significant differences were obtained ($P < 0.05$) between the CFU counts obtained on LNA and the AODC counts, indicating the appearance of a fraction of nonculturable cells. Although the effect of the peroxide was more intense as the concentration of this compound was increased in the water (Fig. 3), in none of these cases did the total number of cells (AODC) decrease.

When compounds which degrade or block the formation of peroxides (catalase, sodium pyruvate, or sodium thioglycolate) were added to illuminated microcosms, the total number of *E. coli* cells (AODC) remained constant throughout the experiment (Fig. 2), as occurred in the illuminated microcosms to which these compounds were not added. Nevertheless, the evolution of culturable cells detected on both of the culture media used showed significant differences ($P < 0.05$) compared with the evolution described for illuminated nonsupplemented microcosms (Fig. 2).

When catalase was added to illuminated microcosms (Fig. 2B), the CFU counts obtained on LNA remained practically constant and statistically significant differences ($P < 0.05$) with respect to initial CFU counts were obtained only at 120 h. The CFU counts on MGA followed an evolution similar to that described for the LNA medium. At 120 h of incubation the CFU counts on MGA were 1 log lower than that obtained on LNA, although this difference was not statistically significant. Thus, the fraction of nonculturable cells increased only at 120 h, as was deduced from the differences ($P < 0.05$) obtained between the number of cells determined by AODC and the CFU counts on LNA medium. Moreover, the fraction of injured culturable cells (CFU counts on LNA minus CFU counts on MGA) did not increase significantly for the same period.

The addition of sodium pyruvate to illuminated microcosms (Fig. 2C) had an effect on *E. coli* cells similar to that described when catalase was added. The reductions observed in the CFU counts on LNA and MGA media did not differ significantly from those detected when catalase was

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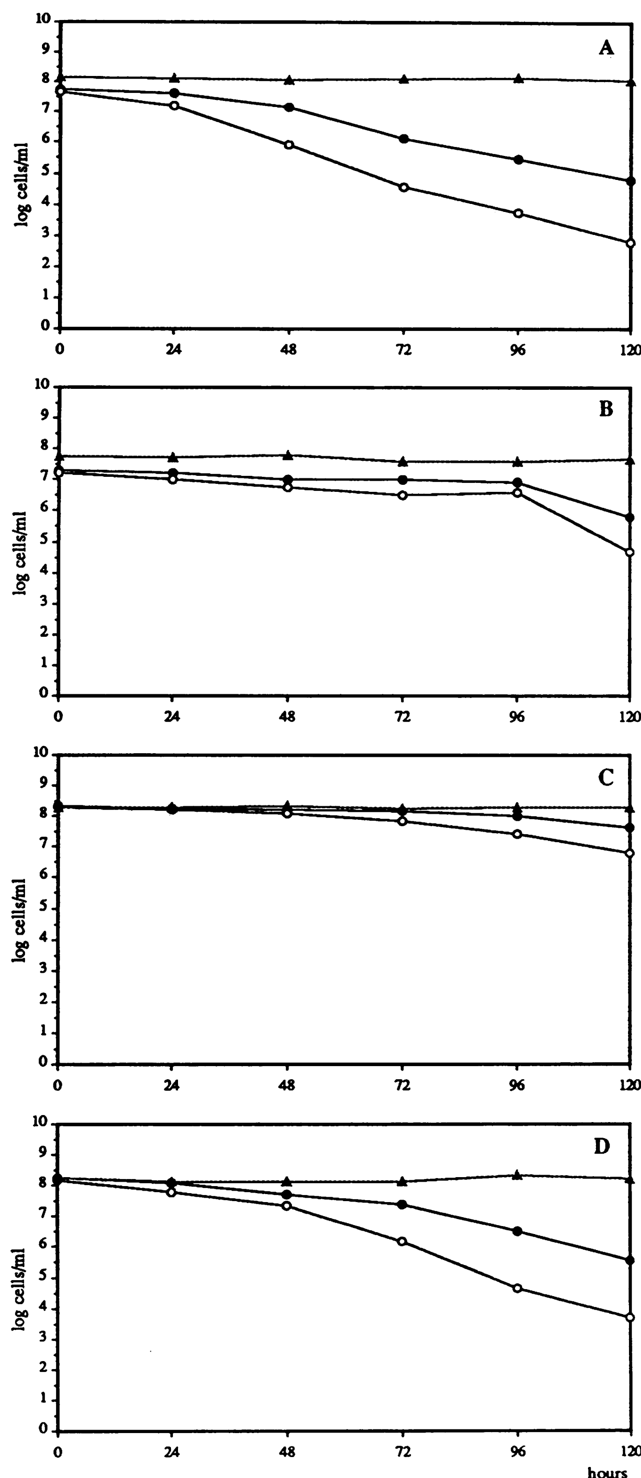

FIG. 2. Cell numbers and CFU counts for *E. coli* in illuminated microcosms. Panels: A, nonsupplemented medium; B to D, media supplemented with catalase (B), sodium pyruvate (C), or sodium thioglycolate (D). ▲, AODC; ●, CFU on LNA; ○, CFU on MGA.

added. Nevertheless, when sodium pyruvate was added, the differences between the bacterial counts at 120 h were not statistically significant.

When thioglycolate was added at 0 and 24 h (data not shown) no differences were observed with respect to nonsupplemented illuminated microcosms. When the addition of thioglycolate was extended up to 72 h and while the addition was maintained, the CFU counts on LNA did not vary (Fig. 2D). However, 24 h after addition of this compound was stopped, the CFU counts decreased and showed statistically significant differences ($P < 0.05$) with respect to the initial number. The CFU counts on MGA remained constant only up to 48 h of incubation and, after this time, decreased significantly ($P < 0.05$). The CFU counts on both culture media showed statistically significant differences ($P < 0.05$) as of 72 h.

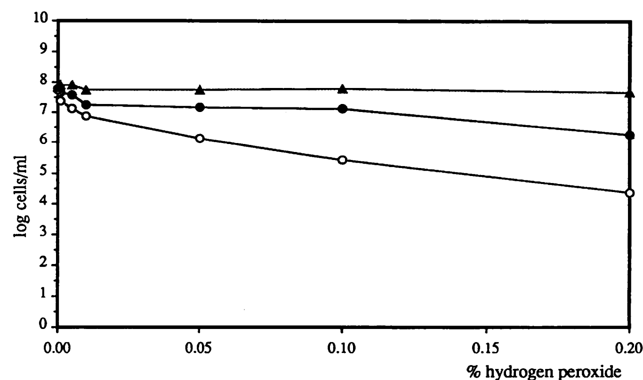

FIG. 3. Cell numbers and CFU counts of *E. coli* obtained after 15 min of contact with hydrogen peroxide. ▲, AODC; ●, CFU on LNA; ○, CFU on MGA.

On the other hand, to prove that the formation of peroxides on the culture media themselves might give rise to cell damage, hydrogen peroxide degraders were added to the culture media. Aliquots from illuminated microcosms and illuminated microcosms supplemented with catalase were spread on LNA and MGA supplemented with catalase or sodium pyruvate. The results of these experiments are shown in Fig. 4. In illuminated microcosms not supplemented with catalase, the addition of catalase or sodium pyruvate to the recuperation medium (LNA) did not significantly increase the number of CFU detected (data not shown). Nevertheless, the peroxide degraders added to the minimal medium (MGA) provided CFU counts significantly higher ($P < 0.05$) than those detected on this medium without either catalase or pyruvate. Besides, the CFU counts obtained on supplemented MGA did not differ significantly from the counts obtained on LNA. In illuminated microcosms to which catalase was added, the supplementing of both the LNA and MGA media with catalase or with pyruvate did not provide CFU counts significantly different from those detected on nonsupplemented media.

DISCUSSION

One of the effects of visible radiation on *E. coli* cells in fresh water is the progressive loss in their capacity to multiply in standard bacteriological media; however, they remain morphologically intact in the natural aquatic medium. Several authors have reported not only the drop in culturable cells (12–14, 17, 23), but also, more recently, the formation of morphologically intact nonculturable cells (2, 4, 5, 10).

Curtis et al. (9) suggest that the apparent effect of light on the bacterial cells is probably due to oxidative stress rather

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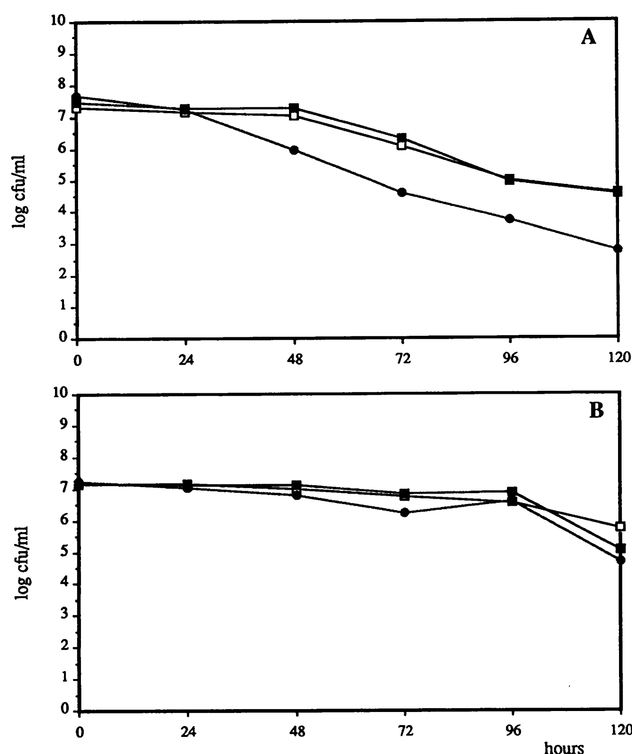

FIG. 4. CFU counts obtained in illuminated microcosms, nonsupplemented (A) and supplemented with catalase (B). ●, MGA nonsupplemented; ■, MGA supplemented with catalase; □, MGA supplemented with sodium pyruvate.

than to light itself. This oxidative stress would be due to the appearance, in illuminated water, of highly reactive oxygen species. From the results obtained in the experiments conducted in order to study the effect of hydrogen peroxide on *E. coli* cells and according to the results of other authors (9, 27), we can deduce that this product has a negative effect on cell culturability. The presence of hydrogen peroxide in the nonilluminated microcosms gives rise to the formation of nonculturable and of injured culturable cells. This negative effect of peroxide depends on its concentration, given that the number of nonculturable and injured culturable cells increases as does the concentration of peroxide in the water (Fig. 3).

Cooper and Zika (8) found that the hydrogen peroxide present in water is mainly photochemically generated from organic constituents and, furthermore, that the accumulation rate of hydrogen peroxide is a function of TOC. These authors, in studies of freshwater ecosystems with TOC values similar to those obtained in our system (from 14.7 to 17.8 mg of C liter^{-1}), detected formation of hydrogen peroxide over a range of 10^{-6} to 10^{-5} M with only 2.5 h of exposure to solar light. In this way, we can suppose that hydrogen peroxide would be photochemically generated in our illuminated microcosms. Thus, the addition at illuminated microcosms of compounds which decompose or block the formation of peroxides (catalase, sodium pyruvate, or sodium thioglycolate) would provide us with information on the evolution of cells in the absence of this product and we could see the effect of other photochemical products and of the direct action of light. The results (Fig. 2) indicate that the elimination of hydrogen peroxide from water has a protective effect on *E. coli* cells given that the number of nonculturable cells and the number of injured culturable cells are lower than those detected in the illuminated nonsupplemented microcosms. Furthermore, the appearance of these cellular fractions (nonculturable and culturable injured cells) in supplemented microcosms was delayed with respect to the illuminated nonsupplemented microcosms. This delay in the appearance of the different cellular fractions allowed us to discard the effect of other photoproducts and of the direct effect of visible light as being the agents which initiated the dormancy process.

When the results obtained in supplemented microcosms are compared, it is observed that the protective effect of catalase and sodium pyruvate remains even when the addition of these compounds is stopped. Nevertheless, in the case of microscosms supplemented with sodium thioglycolate, the continued addition of this compound does not prevent the appearance of injured and nonculturable cells. This different behavior may be due to a greater instability of thioglycolate in our system or to the different mechanisms of actuation blocking the formation of peroxides whereas the catalase and sodium pyruvate degrade previously formed peroxides.

As we said at the beginning of this paper, the use of *E. coli* as an indicator of fecal contamination requires the preparation of new detection methods, this need being more acute because of the culturability loss phenomenon described above. In this regard, Kapuscinski and Mitchell (23) have proposed the supplementation of the minimum culture media, such as MGA, with catalase and/or pyruvate because with this method it is possible to recuperate the injured culturable cells. Thus the CFU counts which they detect in the supplemented minimal medium is equal to that obtained on the recuperation medium, LNA. Our results (Fig. 4) agree with those found by these authors. Nevertheless, the number of cells which are detected on these supplemented media is still very low if we compare it to the number of nonculturable cells presented in the aquatic medium. Thus, the elimination of hydrogen peroxide from culture media does not reduce the fraction of nonculturable cells. We must consider the detection method proposed by Kapuscinski and Mitchell (23) an advance in the quantitative evaluation of *E. coli* cells in natural systems but without forgetting that in the presence of visible light the number of nonculturable cells present in the water could be even greater than that detected by methods which involve culture techniques. In this way, it is important that, as several authors (7, 18, 25, 28, 29) have pointed out, these nonculturable cells maintain their infective capacity.

The results obtained in this work indicate that the hydrogen peroxide formed in water as a consequence of visible light may be one of the photoproducts implicated in the formation of nonculturable and of injured culturable cells in the *E. coli* population. Moreover, to be able to make a quantitative assessment of the total number of *E. coli* cells present in natural aquatic systems, it is necessary to prepare new methods which allow the negative effect produced in cells by hydrogen peroxide to be repaired, thereby allowing nonculturable cells to be detected.

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REFERENCES

1. **Alexander, M.** 1981. Why microbial predators and parasites do not eliminate their prey and hosts. Annu. Rev. Microbiol. **35:**113–133.
2. **Barcina, I., I. Arana, A. Fernández-Astorga, J. Iriberri, and L. Egea.** 1992. Survival strategies of plasmid-carrier and plasmidless *Escherichia coli* strains under illuminated and non-illuminated conditions, in a fresh water ecosystem. J. Appl. Bacteriol. **73:**229–236.
3. **Barcina, I., I. Arana, J. Iriberri, and L. Egea.** 1986. Influence of light and natural microbiota of the Butrón river on *Escherichia coli* survival. Antonie van Leeuwenhoek **52:**555–566.
4. **Barcina, I., J. M. González, J. Iriberri, and L. Egea.** 1989. Effect of visible light on progressive dormancy of *Escherichia coli* cells during the survival process in natural fresh water. Appl. Environ. Microbiol. **55:**246–251.
5. **Barcina, I., J. M. González, J. Iriberri, and L. Egea.** 1990. Survival strategy of *Escherichia coli* and *Enterococcus faecalis* in illuminated fresh and marine systems. J. Appl. Bacteriol. **68:**189–198.
6. **Barcina, I., J. M. González, J. Iriberri, and L. Egea.** 1991. Role of protozoa in the regulation of enteric bacteria populations in seawater. Mar. Microb. Food Webs **5:**179–187.
7. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3:**817–820.
8. **Cooper, W. J., and R. G. Zika.** 1983. Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. Science **220:**711–712.
9. **Curtis, T. P., D. D. Mara, and S. A. Silva.** 1992. Influence of pH, oxygen, and humic substances on ability of sunlight to damage fecal coliforms in waste stabilization pond water. Appl. Environ. Microbiol. **58:**1335–1343.
10. **Davies, C. M., and L. M. Evison.** 1991. Sunlight and survival of enteric bacteria in natural waters. J. Appl. Bacteriol. **70:**265–274.
11. **Flint, K. P.** 1987. The long-term survival of *Escherichia coli* in river water. J. Appl. Bacteriol. **63:**261–270.
12. **Fujioka, R. S., H. H. Hashimoto, E. B. Siwak, and R. H. T. Young.** 1981. Effect of sunlight on survival indicator bacteria in seawater. Appl. Environ. Microbiol. **41:**690–696.
13. **Gameson, A. L. H., and A. J. Gould.** 1974. Effect of solar radiation on the mortality of some terrestrial bacteria in sea water, p. 209–217. *In* A. L. H. Gameson (ed.), Discharges of sewage from sea outfalls. Pergamon Press, Oxford, United Kingdom.
14. **Gameson, A. L. H., and J. R. Saxon.** 1967. Field studies on effect of daylight on mortality of coliform bacteria. Water Res. **1:**279–295.
15. **González, J. M., J. Iriberri, L. Egea, and I. Barcina.** 1990. Differential rates of digestion of bacteria by freshwater and marine phagotrophic protozoa. Appl. Environ. Microbiol. **56:** 1851–1857.
16. **González, J. M., J. Iriberri, L. Egea, and I. Barcina.** 1992. Characterization of culturability, protistan grazing, and death of enteric bacteria in aquatic ecosystems. Appl. Environ. Microbiol. **58:**998–1004.
17. **Grigsby, P., and J. Calkins.** 1980. The inactivation of a natural population of coliform bacteria by sunlight. Photochem. Photobiol. **31:**291–294.
18. **Grimes, D. J., R. W. Atwell, P. R. Brayton, L. M. Palmer, D. M. Rollins, D. B. Roszak, F. L. Singleton, M. L. Tamplin, and R. R. Colwell.** 1986. The fate of enteric pathogenic bacteria in estuarine and marine environments. Microbiol. Sci. **3:**324–329.
19. **Guerrero, R., C. Pedrós-Alió, I. Esteve, I. Mas, D. Chase, and L. Margulis.** 1986. Predatory prokaryotes: predation and primary consumption evolved in bacteria. Proc. Natl. Acad. Sci. USA **83:**2138–2142.
20. **Hagström, A., U. Larsson, P. Horstedt, and V. Normark.** 1979. Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl. Environ. Microbiol. **37:**805–811.
21. **Henis, Y., and M. Alexander.** 1990. Effect of organic amendments on bacterial multiplication in lake water. Antonie van Leeuwenhoek J. Microbiol. **57:**9–20.
22. **Jannasch, H. W.** 1968. Competitive elimination of *Enterobacteriaceae* from seawater. Appl. Microbiol. **16:**1616–1618.
23. **Kapuscinski, R. B., and R. Mitchell.** 1981. Solar radiation induces sublethal injury in *Escherichia coli* in seawater. Appl. Environ. Microbiol. **41:**670–674.
24. **Mallory, L. M., C.-S. Yuk, L.-N. Liang, and M. Alexander.** 1983. Alternative prey: a mechanism for elimination of bacterial species by protozoa. Appl. Environ. Microbiol. **46:**1073–1079.
25. **McFeters, G. A., and A. Singh.** 1991. Effects of aquatic environmental stress on enteric bacterial pathogens. J. Appl. Bacteriol. Symp. Suppl. **70:**115S–120S.
26. **Proctor, L. M., J. A. Fuhrman, and M. C. Ledbetter.** 1988. Marine bacteriophages and bacterial mortality. EOS **69:**1111.
27. **Sammartano, L. J., R. W. Tuveson, and R. Davenport.** 1986. Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli katF* locus. J. Bacteriol. **168:**13–21.
28. **Singh, A., and G. A. McFeters.** 1987. Survival and virulence of copper- and chlorine-stressed *Yersinia enterocolitica* in experimentally infected mice. Appl. Environ. Microbiol. **53:**1768–1774.
29. **Singh, A., R. Yeager, and G. A. McFeters.** 1986. Assessment of in vivo revival, growth, and pathogenicity of *Escherichia coli* strains after copper- and chlorine-induced injury. Appl. Environ. Microbiol. **52:**832–837.
30. **Whitelam, G. C., and G. A. Codd.** 1986. Damaging effects of light on microorganisms. Spec. Publ. Soc. Gen. Microbiol. **17:**129–169.

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