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ENTERIC BACTERIA SURVIVAL FACTORS

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ABSTRACT

In order to improve bacterial water quality of shellfish farming areas, studies were conducted in the English Channel (Morlaix) and on the Mediterranean coast (Toulon). These two areas were chosen in order to compare behaviour of fecal bacteria in two different ecosystems. In the estuary of Morlaix sediments are polluted by way of settlement, but most of the bacteria are mixed with turbid waters and are able to survive a very long time (T90 are in a range of several hours to several days). By measuring the increase in salt tolerance of the strains grown in natural organic matter, it was demonstrated that *Salmonella* can tolerate coastal water salinities. Moreover, because light penetration is prevented by suspended matter, the solar bactericidal effect is very low. On the contrary, through lack of nutrients and very high sunlight intensity, die–off rates in Mediterranean waters are very high (at the surface T90 are less than 2 hours, and several hours in deep waters). A close relationship was found between the light intensity received by bacteria and the T90. Predicted T90 must be found using these two parameters(including turbidity and deep effect on light intensity). The authors suggest that precautions must be taken to carry out impact studies depending on water quality of the area, especially in turbid areas. The knowledge of these mechanisms is very important to evaluate waste water impact on the quality of shellfish farming areas, and to improve elimination of fecal bacteria in sewage treatment plants.

KEYWORDS

Estuaries; bacteria survival; *E. coli*; *Salmonella*; sunlight; salt–tolerance; T90; seawater; sediment.

INTRODUCTION

The enteric bacteria dispersed in coastal marine areas are completely at the mercy of their environment. Depending on temperature, salinity, nutrients concentrations and turbidity, the behavior of these microorganisms will be radically different. Die–off processes were studied on *Escherichia coli* and *Salmonella* on characteristic sites : Morlaix estuary and West Cotentin Coast (on the English Channel) and Toulon on Mediterranean Sea (Fig. 1) ; these areas receive a large bacterial load discharged by sewage treatment plants and rivers. The purpose of this paper is to discuss the conditions under which enteric bacteria die or survive in these different environments depending on sediment characteristics or water quality – i.e. nutrient or organic matter presence and sunlight penetration.

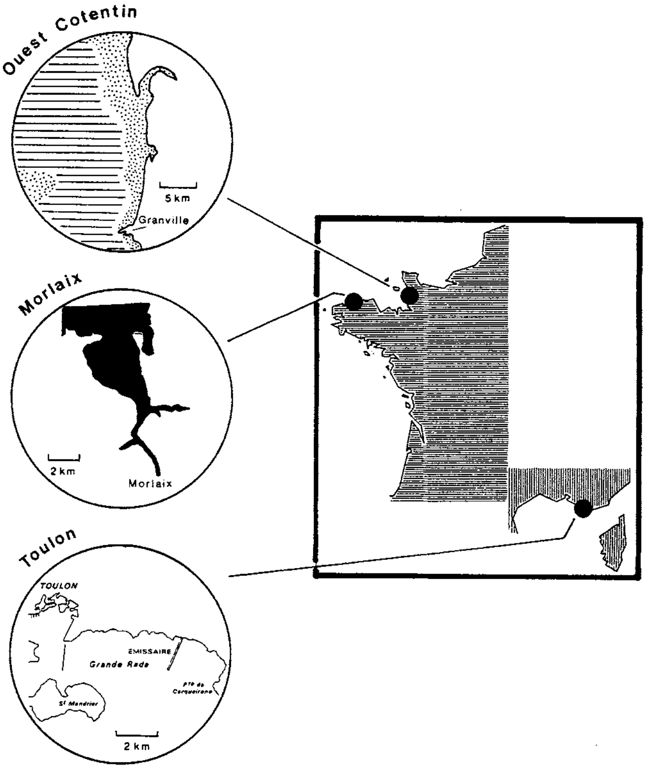

Fig. 1. Location of study areas in Mediterranean sea (Toulon : T) and English Channel (Morlaix estuary : M and West Cotentin Coast : W).

BACTERIAL CONCENTRATIONS

Urban and farm sewage flow into coastal areas and estuaries. These waste waters, which are not always well depurated, contain many bacteria, some of which are fecal and pathogenic for man. After being discharged into the sea, some of these sewage bacteria are diluted and carried seaward.

In fact, mechanisms in estuaries are complex, due to the diversity of bacterial populations ; Plummer *et al.* (1987) clearly indicate that three groups of bacteria occur in estuarine waters : (1) free-living bacteria whose abundance is independent from turbidity ; (2) bacteria attached to permanently suspended particles in water – these are organic large particules ; (3) bacteria able to settle ; the abundance of this last group increases with turbidity.

Free and attached bacteria

Studies on attached bacteria were carried out in Morlaix estuary in order to evaluate the percentage of free or non-free living bacteria in seawater under several hydrodynamical conditions (neap and spring tides) ; samples were collected in sterile bottles at the surface in order to analyse both fecal bacteria and turbidity. The group of attached bacteria was obtained using filtration on Millipore filters (> 3 µm) while free bacteria were provided by the filtrate (< 3µm) and then recovered on 0.22 µm Nucleopore filters, which were incubated at 42 °C on Drigalski medium (Pommepuy *et al.*, 1987) ; the results are presented in figure 2 and show a relationship between the percentage of attached bacteria (adsorbed bacteria/total bacteria) and turbidity (expressed in mg.l^{-1}). The abundance of fecal attached bacteria increases in proportion to the sea water turbidity. The same results were found by Woon Bay Yoon and Rosson (1990) ; Goulder *et al.* (1980) demonstrated that activity of heterotrophic attached bacteria is greater than activity of free bacteria ; attachment is presented as a "strategy" of bacteria to improve metabolism and to protect themselves from zooplankton grazing.

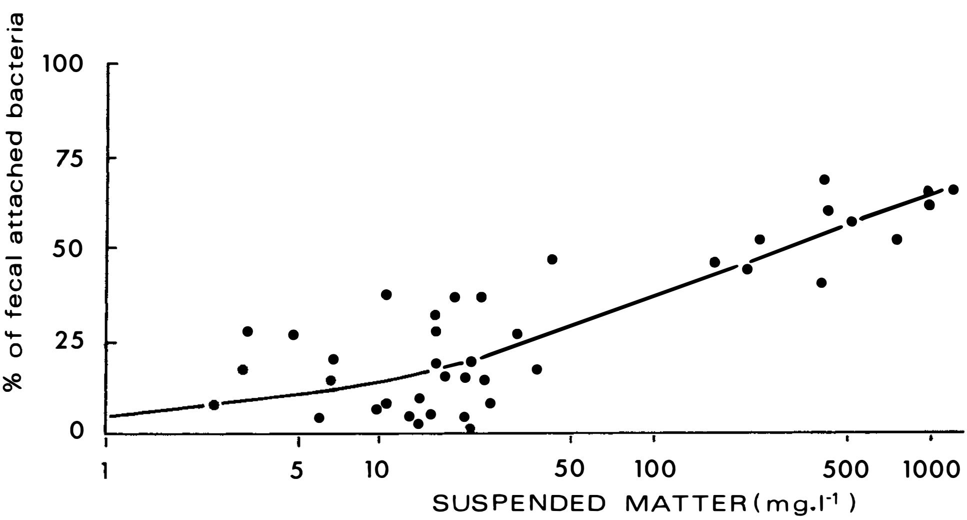

Fig. 2. Relationship between suspended matter (mg l^{-1}) and percentage of fecal attached bacteria. Estuary of Morlaix - Spring tides (June 1987 - October 1987 - March 1988).

Under turbulent flow conditions a zone of relatively still water exists near the bottom (Characklis, 1981) ; in this viscous sublayer bacteria must penetrate in order to be deposited (Marshall, 1985) ; these conditions occur in estuaries and bays.

Fecal contamination in sediments and overlaying waters

Relationships between water and sediment concentrations may be calculated using equivalent units (concentrations are expressed for 100 ml in water and 100 g in wet sediment) ; sampling trips were carried out in June and October (1987) during neap tide and spring tide ; sediments were collected at low tide directly on the surface (top centimeter) in sterile boxes and waters were sampled at low and high tide in sterile bottles. Fecal coliform were analysed using standard methods (filtration or dilution, incubation at 44.5 °C on desoxycholate agar). Results (mean and standard deviation) are shown in figure 3 : each point represents the mean from several analyses.

A significant difference is observed between sediment and water concentrations. In any case fecal coliforms present higher concentrations (1 or 2 log) in the sediment than in the water. These results are in conformity with studies by Erkenbrecher (1981). Sediment fecal coliform concentrations regularly and slowly decrease from upstream (> 10^6 UFC/100 g) – i.e. near sewage treatment plant discharge – to downstream (~ 10^2 UFC/100 g) ; while irregular variations are observed in water. The mean slope in estuarine waters is slightly higher than in sediment due to dilution and bacterial mortality rates.

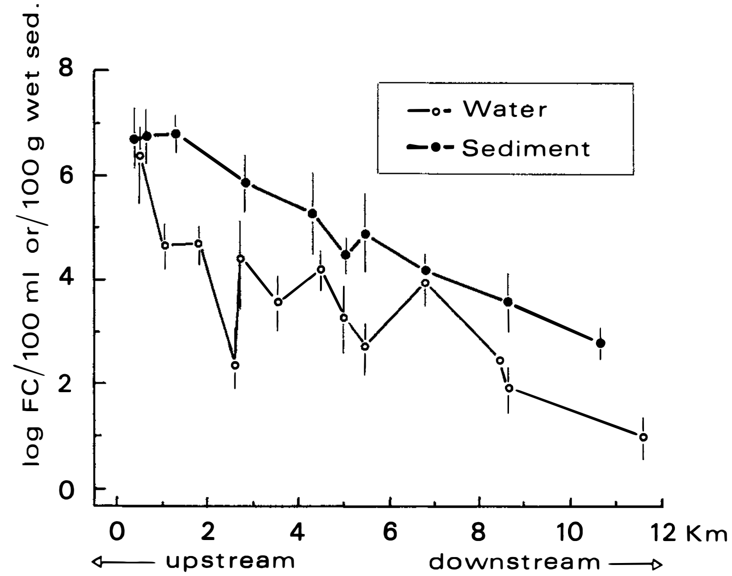

Fig. 3. Evolution of fecal coliform concentration in sediment (●), and in water (O), in Morlaix estuary (mean and standard error).

SURVIVAL IN SEDIMENTS

Behavior of fecal bacteria in sediment

In spite of its high concentration of organic matter, sediment is a limited energy and nutrient environment for microorganisms ; in fact a large part of organic matter is refractory and bacteria cannot always utilize the labile part. Great spatial and temporal variations are observed in terms of nutrient element contributions ; organisms which survive are those which are minimally active, adapted to low available nutrient concentrations, and those capable of very rapid organic matter uptake during short periods of nutrient abundance, and able to survive in inactive states during the long intervening periods (Nedwell and Gray, 1987).

Different alternative strategies may be adopted (Brisou and Denis, 1978). Few bacteria are able to form spores. When external conditions are not highly lethal, microorganisms use different mechanisms to enable themselves to survive. Thus, most adhere to the surface (Janasch and Pritchard, 1972). As demonstrated by Brown *et al.* (1977), bacteria can remain active when nutrient concentrations are very low, which they could not do in the same environment if they were non–adherent ; this also enables them to buffer nutrient compounds more effectively and to be more efficient.

An estuarine survival model for *Vibrio cholerae* has been proposed by Colwell (1983). Survival mechanisms are dependent on organic matter concentrations and available substrates, and require important physiological and structural transformations ; in this case we also observe the appearance of a small–sized cell (*Microvibrio*).

Other steps have also been demonstrated in studies on the survival of *Escherichia coli* (Rosack and Colwell, 1987). In the first "viable" stages (Table I), the bacterium has a metabolism which enables it to be recultured by "soft" methods (preculturing) ; subsequently, cells are no longer culturable but they continue to be able to use exogenous materials and to be metabolically active. Munro *et al.* (1987) observed structural modifications along with enzymatic developments ; the bacteria are considered to be "asleep". In the final stages before death, we can still observe the intact cell by epifluorescence microscopy (Acridine Orange Count).

Direct microscopic examination reveals that such cells account for a large part of the microflora ; the total flora count, as observed by epifluorescence, is 1000 to 10000-fold greater in number compared to the viable heterotrophic flora (Pommepuy *et al.*, 1990).

The survival of bacterial sediment populations can depend on their ability to store energy reserves. The presence of osmoprotectors (glycine-betaine, trehalose, amino acids) may also help to increase survival rates of fecal bacteria in salt environments. Ghoul *et al.* (1989, 1990) observed that when *Escherichia coli* are put into muddy sediments, they are able to intracellularly store exogenous betaine and thus increase their salt-tolerance.

Apparent mortality (T90) in sediment

Many authors have studied survival times of enteric microorganisms in sediment. Sediment protects bacteria from solar radiation, in addition to procuring osmoprotectors and nutrient elements (Gerba and MacLeod, 1976). Survival times are very long for fecal bacteria in sediment. They can vary from several days to several weeks.

The apparent mortality (T90 – time needed for 90 % of bacteria to be unable to cultivate) in sediment, was calculated by Le Guyader (1989) and varies from 6 to 20 days for *E. coli* ; some other groups of bacteria, such as *Salmonella* can survive several weeks in sediments (Gudding and Krodgstad, 1975); the same mortality rates were calculated for fecal streptococci.

TABLE 1 Characterization of cells in survival stages of progressive dormancy (Rosack and Colwell, 1987).

Method of enumeration or detection	Survival at the following stage in the continuum						
	Culturable	Recoverable	Growth responsive	Metabolically active	Dormant	Intact	Dead
Acridine orange direct count	+	+	+	+	+	+	-
Animal-passage recovery	+	+	+	+	+	-	-
Substrate uptake	+	+	+	+	-	-	-
Direct viable count	+	+	+	-	-	-	-
Acclimatization, plate count	+	+	-	-	-	-	-
Standard plate count	+	-	-	-	-	-	-
	VIABLE		SOMNICELL				
	VIVIFORM INCREASING TIME IN SURVIVAL CONDITIONS						

The difference of apparent mortality may be observed in figure 4 between thermotolerant or fecal coliforms (CF) and fecal streptococci (SF).

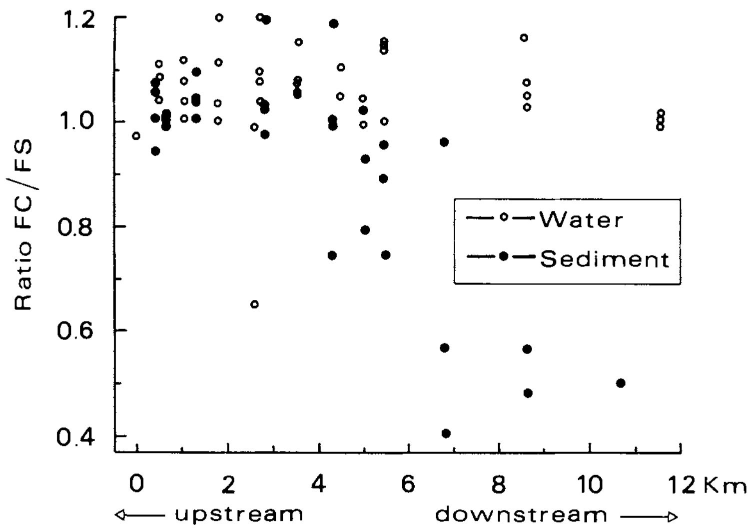

Fig. 4. Evolution of the ratio Fecal coliform/Fecal streptococci (FC/FS) in sediment (●), and water (O), in Morlaix estuary (June and October 1987 - Neap and spring tides).

In sea water where the transit time is very short and often less than one day (Salomon and Pommepuy, 1990), the ratio is slightly higher than 1, while in sediment it decreases from 1 upstream to 0.4 downstream. Upriver from the estuary the bacteria settle, the stable ratio of around 1 is due to recent contamination ; downstream far from the pollution source - it seems that the ratio decrease in sediment must be due to a loss of cultivability of CF greater than that of SF in old polluted sediment. This interpretation is in agreement with results obtained by Pommepuy *et al.* (1990) on the same estuary, where bacterial concentrations function of depth in sediments showed that in deep and old contamined sediment S.F. did not decrease as quickly as C.F. Same results of persistence of fecal streptococci in sediments were found in previous studies from Delattre (1988).

SURVIVAL IN THE WATER COLUMN

The survival mechanisms of enteric bacteria in the water column have been the topic of many studies ; the main factors involved in increasing bacterial survival in the coastal zone are the supply of organic matter as nutrients or osmoprotectors, and the reduction of light's lethal effect by turbidity (Martin and Bonnefont, 1986 ; Crane and Moore, 1986). Temperature also influences the increase or decrease of mortality in estuaries. Rhodes and Kator (1988) working on survival of *E. coli* and *Salmonella spp.* demonstrated that *Salmonella spp.* populations exhibited less mortality and stress than did *E. coli* at low temperatures (< 10 °C).

The role of organic matter on the salt-tolerance

Allochtonous enteric bacteria when entering the marine environment through waste-waters run-off, are subjected to an osmotic shock. To avoid dehydration, cells take up molecules (osmoprotectors) which act as osmotic balancing agents. It has been demonstrated that several compounds can be accumulated intracellularly by *Enterobacteriaceae* (*E. coli, Salmonella, Klebsiella...*) : glycine-betaine (Csonka, 1981), K+ ions and glutamate (Measures, 1975), trehalose (Strom *et al.*, 1986) for the most important.

The ability of bacteria to induce such a mechanism may influence their later survival. Nevertheless, these mechanisms need energy, either for the membrane transport systems activity (K+, glycine-betaine, proline) or for the biosynthesis of osmoprotectors (trehalose, glutamate). In environments with large amounts of organic matter, bacteria could find both nutrients and osmoprotectors. Under this hypothesis, we studied increase of the salt-tolerance of *Salmonella* induced by estuarine waters. 18 strains of *Salmonella* were grown in sterile estuarine water (sampled in the Morlaix estuary), added to the mineral compounds of a minimal medium M63, at serial NaCl concentrations. Medium M63 made with distilled water was used as the control. We measured growth in each case, after 5 days at 15 °C, and 1 day at 37 °C. The difference showed the increase of the salt-tolerance induced by organic matter. The same experiment was carried out with filtered (0.22 µm) waters, in order to determine the respective roles of dissolved and particulate organic matter. Figure 5 shows that the greatest amount (23.5 mg l^{-1} : water A) yielded a 10 g l^{-1} NaCl increase of salt-tolerance for 89 % of the strains (at 37 °C) and for 72 % (at 15 °C). Whereas the second tested water (12.4 mg l^{-1} : water B) allowed the same increase for only 22 % of the strains. These results are in good agreement with our previous study (Pommepuy *et al.*, 1991).

The dissolved organic matter appeared to be the most effective (fig. 5), most likely because it is the most easily assimilated, either at 37 °C or at 15 °C. Even if a large amount of environmental organic matter increases the salt-tolerance of allochthonous bacteria, it must be kept in mind that its composition and nature are the most important factors.

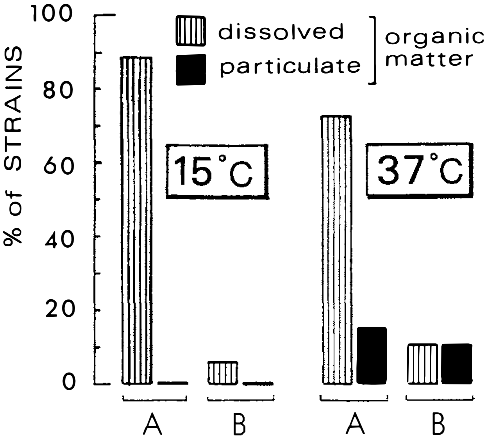

Fig. 5. Percentage of *Salmonella* strains showing a 10 g l^{-1} NaCl increase of their salt-tolerance when grown with the organic matter of two different estuarine waters (A and B).

The role of light attenuation

For a long time, light has been recognized as a die-off factor for enteric bacteria in sea-water (Gameson and Gould, 1975) ; however in the coastal zone, light attenuation by turbidity is responsible for an increase in bacterial survival.

In order to evaluate this phenomenon, field measurements of light attenuation and bacterial survival were carried out in the English Channel (Morlaix estuary and West-Cotentin coast) and in the Mediterranean Sea (Bay of Toulon - Bonnefont *et al.*, 1990).

Light intensities were measured at different depths with a submersible quantum sensor (LICOR) in the wave length band from 400 to 700 nm ; the vertical attenuation coefficients were calculated according to the Lambert law : $I = Io\ e^{-kz}$ (where Io is light intensity at the water surface, I is light intensity at depth z, and k is the attenuation coefficient).

The part played by dissolved and particulate matter in light attenuation was evaluated by laboratory measurements of light transmission with a spectrophotometer UVIKON 810 (wave length band : 400 to 700 nm). The attenuation coefficients were calculated for raw water samples (kr) and for filtered water samples through a 0.45 μm membrane filter (kf) ; the difference between the two measures gives the attenuation for suspended matter (ks = kr − kf).

Field bacteria survival experiments were carried out using quartz chambers (volume : 600 ml) closed by 0.45 μm Millipore membranes. For full details of sampling sites and bacterial method see Pommepuy *et al.* (1990). One ml of *E. coli* suspension was initially introduced into the chambers, which were located at different depths in the water colum. Aliquots were periodically withdrawn and numerations were carried out using a standard membrane filter technique with incubation on Drigalski agar medium (Pommepuy *et al.*, 1987). A simple first-order model was used ($dc/dt = -\mu c$) to estimate the bacterial disappearance rate (μ in h^{-1}) and T90, which is the time (in h) needed for 90 % of bacteria unrecoverable by culture ; it is closely linked to μ by the equation $T90 = 2.3/\mu$.

Results concerning field measurements of visible light attenuation confirm that there is a good relationship between suspended matter concentrations and light attenuation coefficients (fig. 6) ; this figure shows that in turbid estuarine waters (S M ~ 100 mg.l^{-1}) light transmission through the water column could be reduced by a factor greater than 10, in comparison to clear coastal waters (S M between 1 and 5 mg l^{-1}).

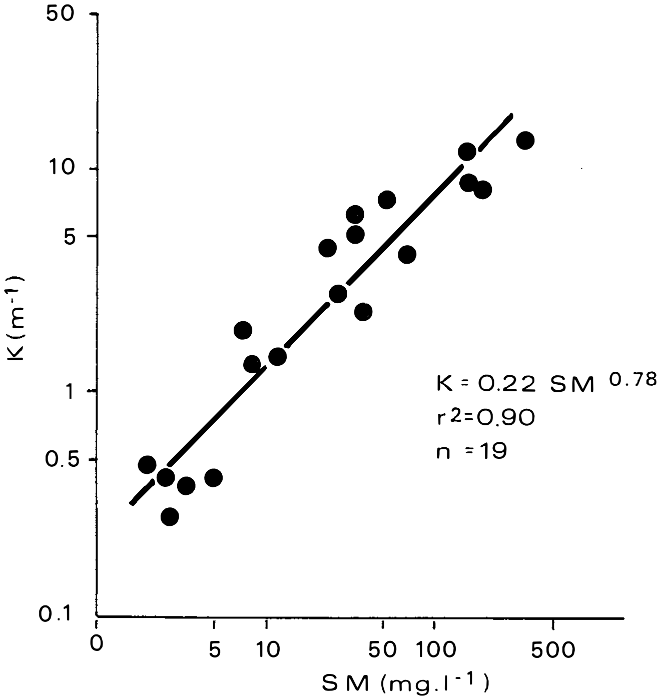

Fig. 6. Relationships between attenuation coefficient (k) and suspended matters (SM) in Morlaix estuary.

The laboratory evaluation of the percentage P of light attenuation by suspended matter ($P\ \% = 100\ ks/kr$) shows that in estuarine waters of Morlaix, the particulate matter is responsible for about 90 % of the light attenuation when suspended matter concentration is greater than 10 mg l^{-1} (fig. 7). In seawater, where suspended matter concentration is less than 5 mg l^{-1}, this percentage P is still between 60 and 80 % ; in the fresh riverine water, dissolved organic matter also contributes to light attenuation and the proportion P of attenuation by suspended matter is about 73 %.

These results show that, in the coastal zone, the main factor of light attenuation is particulate matter, resuspended by waves or tidal currents.

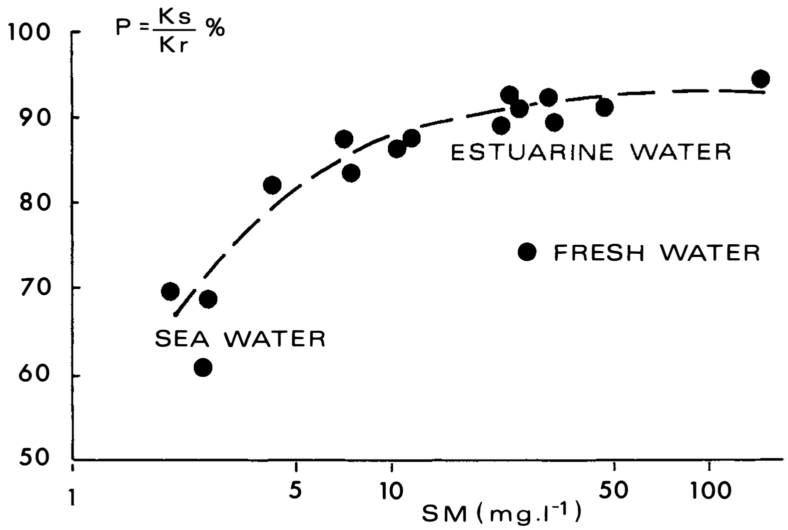

Fig. 7. Percentage of light attenuation by suspended matter – Morlaix estuary.

Results of bacteria survival experiments carried out in the English Channel and on Mediterranean coasts enable the calculation of a relationship between T90 and light energy received by bacteria at different depths in the water column (fig. 8). This relation is close to the one found by Bellair *et al.* (1977) and it shows that T90 increases when light energy is weak ; this process seems to be the same in the two kinds of coastal zones.

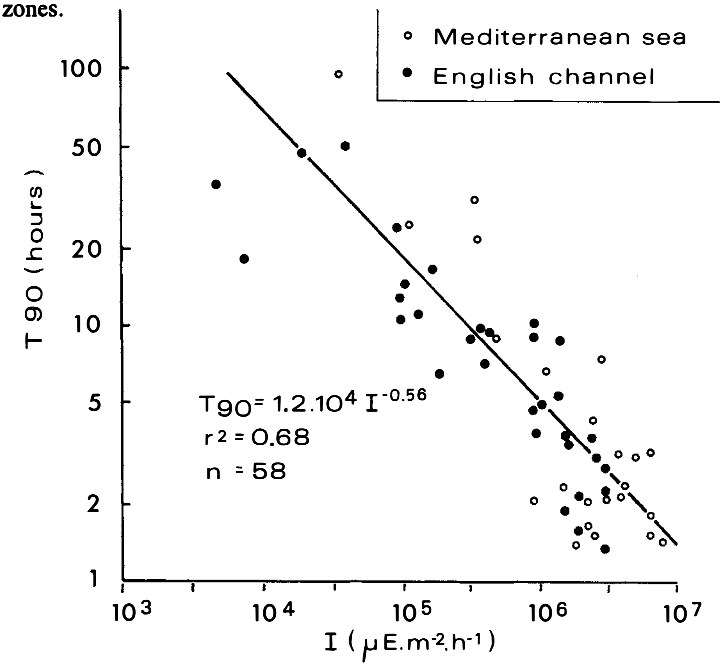

Fig. 8. Relationship between T90 and light energy in the coastal areas.

Nevertheless in the Mediterranean environment, the clearness of water induces, during sunny day, T90 values between 1 and 5 h for subsurface waters ; in the English channel, for the same conditions of incident light and depth, T90 can be about 50 h because of high turbidity of water, especially in an estuarine environment.

This study leads us to take account of turbidity conditions for the estimation of bacterial survival and impact assesment in case of waste-water disposal in the coastal zones (Pommepuy *et al.*, 1991).

CONCLUSION

The behavior of fecal bacteria is closely dependent on the environment's water or sediment quality. In coastal areas, fecal bacteria and pathogenic bacteria as *Salmonella*, are able to find necessary elements for life : nutrients and osmotic compounds enable them to endure high salinity. On the other hand, suspended matter drastically impairs the visual clarity of water and therefore, by light scattering, protects bacteria from a bactericidal sunlight effect.

On Mediterranean coasts, oligotrophic water and high solar radiation due to the climate greatly diminish the survival time of fecal bacteria in surface water : T90 are very short (< 2 hours) at the surface ; but in deep waters, in the same region, where the wastewaters plume is trapped under the thermocline, the T90 could be longer (several tens of hours).

In the English Channel, waters and sediments are rich in organic matter ; in addition the cloudy system and high turbidity significantly increase fecal bacterial survival : T90 may be very long, from several tens to several hundreds of hours.

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