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THE VIABILITY OF *E. COLI* IN SEA WATER AT DIFFERENT TEMPERATURES

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Abstract

The accidental multiplication of conditional pathogen bacteria such as E.coli in the territorial sea waters of Romania has an important impact on human health and requires a continuous evaluation of its risks. This study is focused on the effect of some physical factors (temperature, salinity, nutrient concentration) on the viability of Escherichia coli cells. In our experiments, we analysed the response of E.coli grown in normal conditions (LB at 37 ^{0}C) when subjected to a nutritional, saline and osmotic shock by introducing sub-samples of the culture in three (4°C, 15°C, 37°C) separate sterile sea water (sea water filtered through a Millipore filter- pores of 0.22 µm) microcosms. The samples were collected at different times (ranging from minutes to days) and analysed with respect to total cell densities (cells permeable to Syber green), dead cells (cells permeable to propidium iodide) densities and colony forming units. The results showed that the stress conditions determined by osmotic shock, saline shock and nutrients deprivation cannot suppress the resistance and multiplication capacity of E.coli after two days, even though the stress conditions diminished them. Although the temperature of the sea water is an important factor regarding the resistance of E.coli, a rather large proportion of cells continue to remain alive at 4°C, and able to multiply when transferred to LB at 37°C.

Key words: *E.coli, starvation, microcosm, Black Sea, SYBER Green, propidium iodide, colony forming units.*

INTRODUCTION

Due to the impact on human health and marine life the multiplication of conditional pathogen bacteria such as *E.coli* in the territorial waters of the Black Sea is and will be a continuous risk that needs permanent evaluation. Worldwide researchers try to evaluate the risk of exposing *E.coli* in the sea waters; risk that implicate not only the human populations but also the marine macroorganisms and microorganisms. (Greenberg, 1956; Carlucci and Pramer, 1960; Lessard and Sieburth, 1983; Gonzales et al., 1992; Davies, et al., 1995). Based on literature (Carlucci and Pramer, 1960; Carlucci et al., 1961; Gameson and Gould, 1975; Anderson et al., 1979, 1983; Fujioka et al., 1981; Welch et al., 1993; Ingraham and Marr, 1996; Troussellier, 1998; Ghita and Ardelean, 2011) it is well known that physical factors like temperature, salinity, pH, light radiation and nutrient availability play a key role in the survival of sea water bacteria, in particular potential pathogen bacteria. Nutrient depravation and light radiation associated with oxidative stress are belived to be most the most hostile stress factors of shallow costal sea waters.

This paper is focused on the time evolution of *E coli* alive cells, dead cells and cells able to grow and divide following their passage from LB medium (37° C) to sea-water kept at different temperatures (4°, 15° and 37° C).

MATERIALS AND METHODS

Strain

E.coli (non pathogenic strain) was grown in LB 37°C on a orbital shaker (150 rpm) and collected after 18 hours of growth. The cells were aseptically washed two times in sterile phosphate buffer saline to eliminate organic substrate, and further quickly introduced in indoor microcosms.

Sampling

The sea water used in this experiments was collected from the territorial waters of the Black Sea at 1 m depth (Constanta). The

water filtrated into sterile bottles by using 0.22 μm Millipore to avoid the inclusion of bacteriovorus microorganism and also other bacterial competitors. Many studies show that protozoa represent the main predators of *E.coli* populations in sea waters. (Gonzales, 1992; Barcina et al., 1992; Enzinger and Cooper, 1976; Sherr and Sherr, 2002).

Microcosms

We constructed three microcosms that each consisted of 1 L filtered see water in which it was added 0.5 mL *E. coli* sub-culture. The microcosms were kept in the dark at 4°C, 15°C and 37°C for 48 hours. The samples were collected immediately after inoculation and at 1h, 3 h, 22 h, 28 h and 48 h.

Total cell count

SYBER Green was used a fluorochrome to label all cells, both alive and dead (Figure 1) as it permeates both cells with intact and functional plasma membrane (living cells) and cells with altered plasma membrane (so called dead cells) (Lunau et al., 2005) The samples were filtered through polycarbonate Nuclepore filters with Millipore funnel as previously shown (Lunau et al., 2005; Manini and Danovaro, 2006; Ghita and Ardelean, 2011). In order to count bacteria, 300-600 cells were counted on each filter (usually 10-20 microscopic fields) and special attention was accorded to the filtration process in order to achieve an as uniform as possible distribution of bacterial cells all over the filtration surface (Sherr and Sherr, 2002). The average density of cells was converted to cell densities following appropriate equation (Cell density/mL = { [3,14 x (75000x75000)] :[3,14x(11,5x11,5)] }x number of counted cell/field).

Dead cell count

The same samples were stained with propidium iodide (Figure 2), a commonly dye used to differentiate necrotic/apoptotic, cells. Propidium iodide is membrane impermeant and is excluded from viable, non-apoptotic cells. (Moore et al., 1998; Manini and Danovarro, 2006).

Cells able to grow and multiply

The determination of cells able to grow and multiply was done by quantification of the colonies formed on LB agar at 37 C using droplet method (Neblett, 1976; Hoben and Somasegaran, 1982). Fresh subsamples of 10 μl each were collected and inoculated letting the colony forming units to grow for 12-24 hours at 37°C. For a better view of the colonies they were stained with basic fuchsine (0,004% w/w) before counting (Figure 3).

Figure 1. *E.coli* stained with SYBER-green

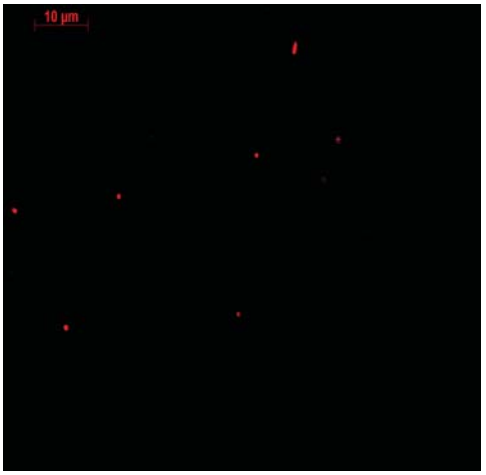

Figure 2. E.coli stained with propidium iodide

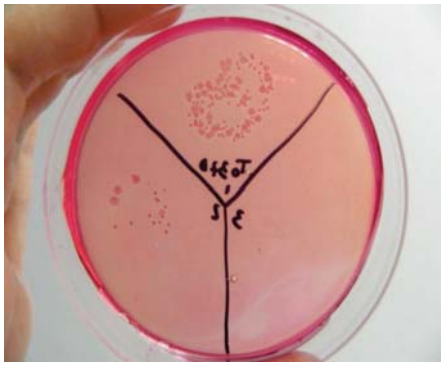

Figure 3. E.coli colonies stained with basic fuchsin 02004%.

RESULTS AND DISCUSSIONS

In figures 4,5 and 6 there are presented the results concerning the time evolution of alive E coli cells (SYBER green permeable cells minus propidium iodide permeable cells), dead cells (propidium iodide permeable cells) and cells able to grow and multiply (colony forming units) respectively. As it is mentioned in the literature, temperature plays a key role in the survivability of *E.coli* and also on its capability of forming colonies. Various studies claim that the optimal temperature for survival of *E.coli* is different and notable lower than the temperature optimal for its growth (Carlucci and Pramer 1960; Carlucci et al., 1961; Vasconcelos and Swartz, 1976; Lessard and Sieburth, 1983).

Although there was a similar tendency between the samples collected from the 4°C microcosm and the one held at 15°C, there was no substantial difference. As shown in the first graphic the number of live cells kept at 4°C seems to be slightly higher. Regarding cells able to grow and multiply, on the other hand, the difference between these two temperatures, was significant with respect to the 4°C. The population of *E.coli* kept at this temperature contains a lower number of cells able to grow and multiply than the population kept at 15°C (Figure 6).

The state of viable but nonculturable cells was proven to be, in different studies, a state in which cells preserve metabolically active though being unable to divide on nutritional media specific for theirs growth (Roszakt and Colwell, 1987; Pommepuy et al., 1996).

Nevertheless, as seen in the figures 4, 5 and 6 even after only a few hours, although the density of viable cells in not radically diminished, the density of cells able to grow and multiply is rather largely affected at lower temperature (4°C).

Taking into account these results, one must assume that in the summer period, when the temperature is higher, an outbreak of potential pathogen bacteria in the territorial waters of the Black Sea is indeed possible. Though it's true that *in situ* there are other factors such as UV radiation, hydrostatic pressure and other concurrent bacteria, bacteriovores and microorganisms of the sea to be taken into account in further studies.

Also it is notable that as mention in different studies (Anderson et al., 1983) the temperature of 4°C, even after 48 hours can not totally suppress the ability of *E.coli* populations that can survive at this temperature; in agreement with the results presented in this paper, a small fraction of cells continue to remain viable, and able to multiply when transferred to LB at 37°C.

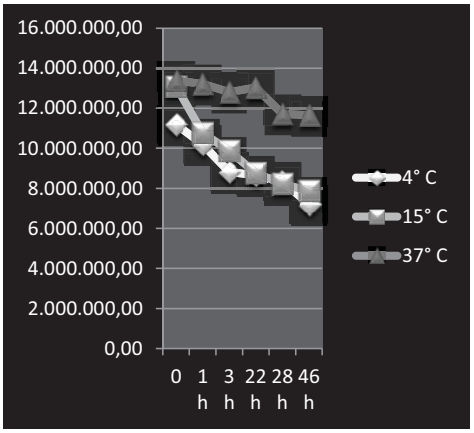

Figure 4. Time evolution of alive/live E coli cells densities (total cell -SYBER Green positive- minus dead cells -propidium iodide positive) in filtered sea water, at different temperatures.

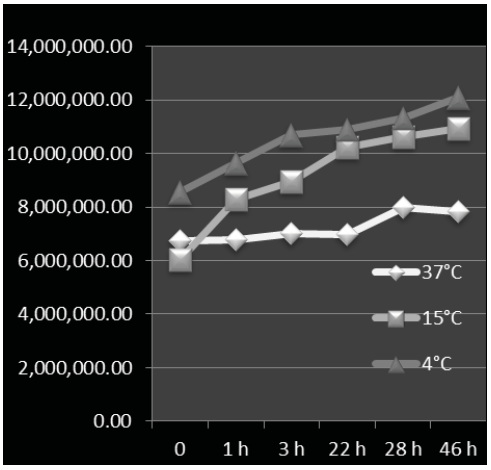

Figure 5. Time evolution of dead cells densities (propidium iodide positive) in filtered sea water, at different temperatures.

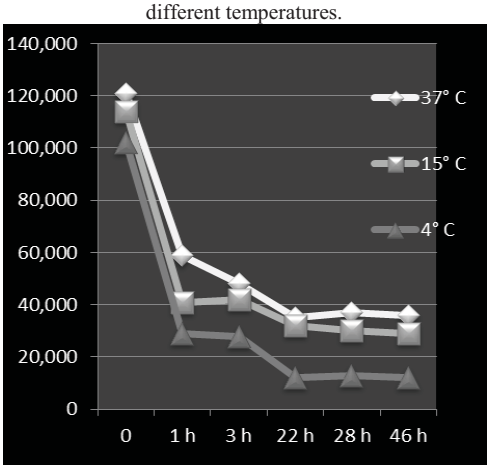

Figure 6. Number of cells able to grow and multiply in LB medium after the cells have been kept in filtered sea water at different temperatures.

CONCLUSIONS

1. In time, there is a linear decrease in the density of living cells total cell (SYBER-green positive) minus dead cells (propidium iodide positive) and a corresponding increase in the density of dead cells (propidium iodide positive).
2. The densities of cells able to grow and multiply (colony forming units) decrease very sharply (orders of magnitude) in the first hours after the beginning of the experiment, the decrease being higher at lower temperatures.
3. Total cell count (SYBER- green labelled cells) of *Escherichia coli* in filtered sea water remain constant all over this short term experiment (48 hours), suggesting that, in these experimental conditions, cell multiplication or physical dissolution do not occur.

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