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THE DYNAMICS OF LIVE AND DEAD CELLS, AND COLONY FORMING UNITS OF *E. COLI* DH5 α STRAIN KEPT FOR FIVE DAYS AT 18°C IN MICROCOSMS CONTAINING FILTERED AND UNFILTERED SEA WATER

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Abstract

The aim of this study is to determine the time-evolution of live and dead cells as well as the number of colony forming units of E. coli strain DH5α after the passage from LB medium to seawater indoor microcosms. In order to determine the dynamics and survivability capacity with respect to the absence or presence of bacterivores microorganisms E. coli sub-samples were housed in three different microcosms containing filtered (0.22 or 0.45 µm pores) and unfiltered sea water. The microcosms were kept for five days in a sterile environment at a constant temperature of 18 °C in the dark. The samples collected were examined by fluorescent microscopy (SYBER green and Ethidium homodimer) for the determination of cell density (total /dead) and colony forming units quantification (LB and Levine media) in order to establish C.F.U. growth. The results presented in this paper show that the shocks associated with the passage from LB medium to sea water indoor microcosms (hypothermic, hypo-osmotic, including low levels of nutrients, and the presence of bacterivores microorganisms) play a key role in the survival of E. coli cells in this five days period.

Key words: *E. coli, live cells, colony forming units, protozoa predation, microcosm.*

INTRODUCTION

During the last decades the Black Sea waters have suffered multiple changes due to pollutant discharge. Human interventions and hydraulic regime of the rivers played an important role in the changes of the phytoplankton, zooplankton and zoobenthos (Bologa et al., 1995; Bakan and Buyukgungor, 2000).

Similar unsteadiness in sea waters worldwide has driven researchers to study the harmful contingent risks of multiplication of potential pathogenic bacteria such as *E. coli* (Greenberg, 1956; Lessard et al., 1983; Davies et al., 1995). Numerous studies show that changes in sea water like high temperatures due to global warming, salinity, nutrient availability, light radiation and protozoa predation influences the survival of potential pathogen bacteria (Carlucci et al., 1961; Gameson and Gould, 1975; Fujioka et al., 1981; Anderson et al., 1983; Ingraham and Marr, 1996; Trousseller, 1998).

Taking all into consideration, we felt that a study that features the current stage of the Black Sea waters particularities regarding potential pathogenic cell multiplication is necessary and important in order to monitor the evolution of bacteria such as *E. coli* that had accidentally reached sea water by shedding and polluted sewage.

The aim of this paper is to monitor the dynamics of *Escherichia coli* cells (live, dead) and their ability to grow and multiply after being kept in three different microcosms containing filtered (0.22 or 0.45 µm pores) and unfiltered sea water at 18 °C for five days.

MATERIALS AND METHODS

Strain and Sampling

We used a nonpathogenic strain of *E. coli* (DH5α) that was previously grown in LB 37 °C on an orbital shaker (150 rpm). The cells were collected after 18 hours of growth and were aseptically washed two times in sterile phosphate buffer saline to eliminate organic substrate.

The sea water was collected in the autumn from the Black Sea territorial waters (Constanta) at 1 m depth. The water was filtrated into sterile bottles using either 0.22 µm Millipore or 0.45 µm Millipore filters.

Microcosms

Three microcosms were housed in sterile 1 L bottles. The first one contains sea water filtered by 0.22 µm in order to assure a sterile environment by eliminating all other cells, including different types of bacterivores pre-existing in the water. The second microcosm contains 0.45 µm filtered water to avoid the inclusion of bacterivores microorganism, but allowing the presence of other small microorganisms; the third one was with unfiltered water, a configuration more similar to the natural conditions. As many studies show that other concurrent bacteria and protozoa predation play an important role in the survivability of *E. coli* population, (Enzinger and Cooper, 1976; Barcina et al., 1992; Gonzales et al., 1992; Sherr and Sherr, 2002) the choice of having three different microcosms with the particularities described above is fundamentally important, in order to establish the ability of *E. coli* cells to remain intact and to keep the capacity to grow and multiply after reaching the sea water. In each of the three microcosms 0.5 mL *E. coli* DH5α sub-culture previously adjusted to an OD =1.0 was added. All were kept for five days in the dark at a constant temperature of 18 °C. The samples were collected immediately after inoculation (time zero), and also at 1 hour, 3 hours, 22 hours, 46 hours, 3 days, 4 days and 5 days.

Enumeration of live and dead *E. coli* cells

The samples were analyzed in order to assess the viability of the *E. coli* populations and also their capacity of multiplication under the environmental stress of each microcosm. Fluorescence microscopy was used to determine the cell densities in order to ascertain the number of remaining live *E. coli* cells in each of the three microcosms. The samples were stained with SYBER green for labelling the DNA of all the *E. coli* cells, the living cells, with intact and functional plasma membrane (live cells), and also cells with altered plasma membrane (dead cells) (Figure 1a). We also used Ethidium homodimer as a membrane impermeable dye to stain the same samples in order to highlight dead cells) (Figure 1b), cells with plasma membranes that have an unphysiological permeability, allowing the passage of large, negatively charged molecules (Manini and Danovaro, 2006).

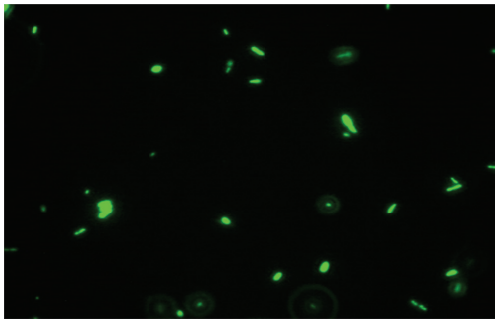

Figure 1a. *E. coli* cells stained with SYBER-green

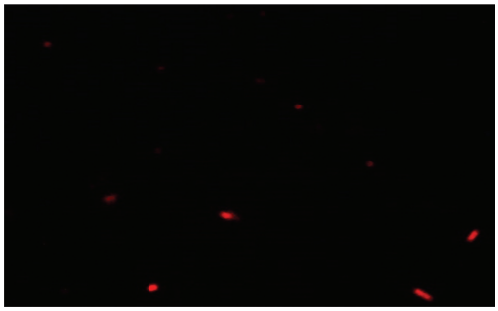

Figure 1b. *E .coli* cells stained with Ethidium homodimer (the same microscopic field as in Figure 1a)

For the fluorescence microscopy the samples were filtered through polycarbonate Nucleopore filters with Millipore funnel attempting an uniform distribution of bacterial cells over the filtration surface. The quantification was realized for a media of 15 microscopic fields for each probe and these readings were realized with Ob.100x, oil immersion. The cell density for each probe was determined with the following formula (Manini and Danovaro, 2007):

Cell density/ml = { [3,14 x (75000x75000)]:(46 x 98) }x number of counted cell/

First the number of total cells (SYBER-green labeled) was calculated and then in parallel the number of dead cells (Ethidium homodimer labeled). By deducting the number of dead cells from the total cell number for each microscopic field the number of live cell was obtained; these numbers were further used to calculate the density of live cells per volume (mL).

Quantification of colony forming units

In order to establish the ability of the *E. coli* cells to grow and multiply after being kept in these three microcosms, the number of colony forming units was determined. Towards avoiding any interference from the marine bacteria present in the 0.45 µm filtered microcosms and the unfiltered one, the samples were inoculated on two different culture media: LB and Levin (Figure 2a and Figure 2b). Although we have used before in our similar experiment Luria-Bertani culture medium as a specific media for coliform bacteria, adding a highly specific medium such as Levin in order to highlight the colonies of *E. coli* was mandatory (Cristina and Ardelean, 2015).

From each probe 10 µl were inoculated by using the droplet method (Neblett, 1976; Hoben and Somasegaran, 1982). The plates were then incubated at 37 °C for 24 hours.

RESULTS AND DISCUSSIONS

By maintaining all three microcosms in the same constant environmental parameters (18°C, darkness) we assured that the differrences between the evolutions of the *E. coli* populations relies only on the dissimilarities of the filtration of the sea water, namely the absence or the presence of bacterivores microorganisms.

Regarding the evolution of live cells density in these five days of study we argue that the nutritional competition and protozoa predation plays an important role in the survival of *E. coli*. As it is notable in the following graphic (Figure 3) the number of live cells decreases considerable in the unfiltered sea water microcosm with respect to the first two microcosm systems (the one with sea water filtered through 0.22 µm Millipore filters and the one filtrered through 0.45 µm Millipore filters). Like many studies show, protistis such as some dinoflagellates, including those from the Black Sea may act as predators for bacteria (Kofoid and Swezy., 1921; Barker, 1935; Schnepf and Elbrächter., 1992). In our five days experiment the number of total cell count from the unfiltered seawater microcosm decreases drastically, as does the number of live cells (Figure 4).

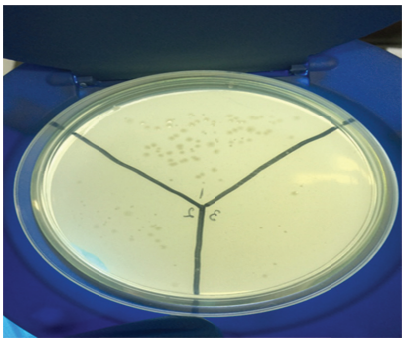

Figure 2a – *E. coli* C.F.U. on LB culture media

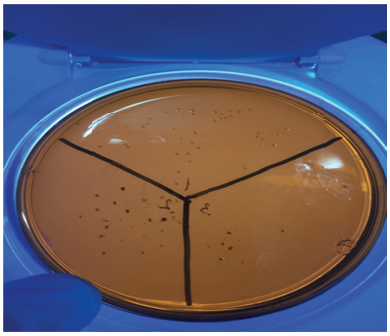

Figure 2b – *E. coli* blue-metalic C.F.U. on Levine culture medium

However, the number of total cell count (SYBER-Green labelled cells) from the other two microcosms, with filtered sea water, is maintained relatively constant, suggesting, as it has been previously demonstrated, that for short periods of time cell destruction does not occur (Cristina and Ardelean, 2015).

The loss of noteworthy cells due to the contingent formation of biofilms was not taken into consideration as *E. coli* DH5α strain used in this experiment expresses poor mobility and cell attachment, which are essential for biofilm formation (Wood et.al. 2006).

The number of dead cells increases sharply within the five days period in each of the three microcosms (Figure 5). Loss of cell integrity when submerged for different periods of time in sea water microcosms systems was described for *E. coli* strains in other studies as well. As it has been previously described, the osmotic stress suffered by *E. coli* population cells when submerged by direct inoculation from the culture in sea water microcosm determines a high mortality rate from the first

hour (Cristina and Ardelean, 2015; Omrane et al., 2011).

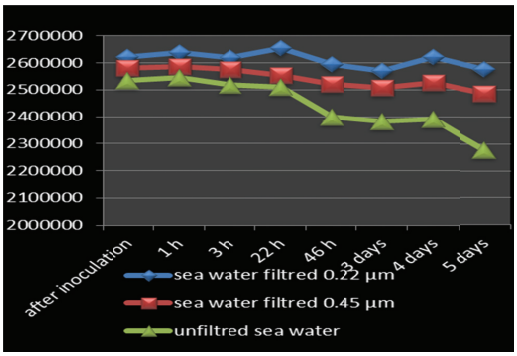

Figure 3. The evolution of total cells count during the five days experiment (SYBER Green *E. coli* labelled cells) in each of the three microcosms systems at 18 °C

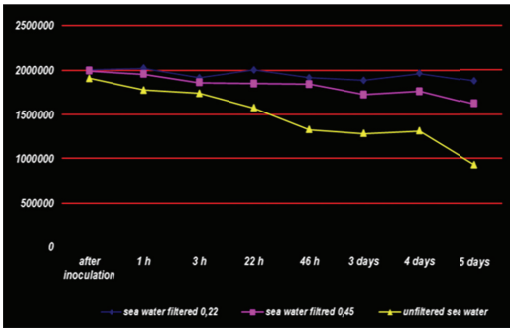

Figure 4 – The evolution of living cells during the five days experiment (SYBER Green *E. coli* labeled cells minus Ethidium homodimer – labeled cells) in each of the three microcosms systems at 18 °C

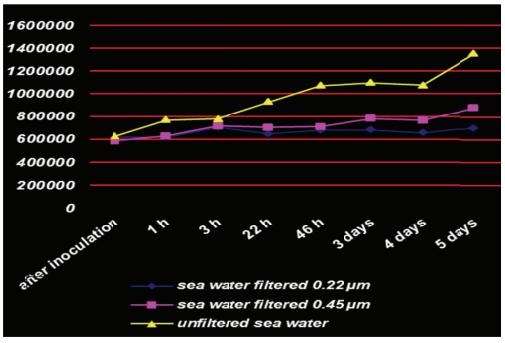

Figure 5 - The evolution of dead cells during the five days experiment (Ethidium homodimer – *E. coli* labelled cells) in each of the three microcosms systems at 18 °C

On the other hand, the stress factors of marine environment induce a loss of the colony forming ability although the cells maintain their viability (Figure 6a and Figure 6b). We can assume that this fact is due to their capacity to converge in a viable but nonculturable state. The nonculturable state, as explained in different studies is a state in which cells, though viable, are unable to divide on nutritional specific media (Roszakt and Colwell, 1987; Pommepuy 1996). The multiplication capacity does not vary substantially between the microcosm with sea water filtered 0.22 µm and the microcosm with sea water filtered 0.45 µm on the five days period of our study. By difference the culturable state of the *E. coli* cells from the microcosm with unfiltered sea water suffer a notable decrease from the 22 hours point on. After four days there was not any C.F.U. growth among the probes from the unfiltered water microcosm on culture medium, LB, or Levin. We repeated these probes in order to present an accurate result. Taking this into consideration and also due to the fact that there was a sharp decrease of C.F.U. in both media, LB and Levine, after five days in the unfiltered sea water microcosm, we could argue that the nutritional competition of the sea water microorganisms but mostly the activity of bacterivores is crucial to the survivability of *E. coli*.

Though not as dramatically, the number of *E. coli* C.F.U. from the microcosm with filtered sea water (0.22 µm) decreased linearly through the whole time period of the experiment. As proven in other experiments as well, the reduction of *E. coli* cells cultivability when submerged in seawater is substantially even in a five days period (Omrane et al., 2011). The cells from the microcosm with sea water filtered through 0.45 µm undertake a similar pattern.

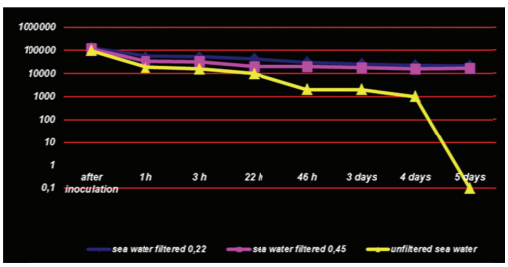

Figure 6a – The evolution of colony forming units of *E. coli* cells in each of the three microcosms systems at 18 °C, after inoculation in LB culture medium, during the five days experiment

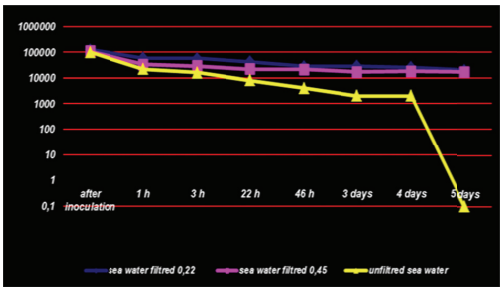

Figure 6b –The evolution of colony forming units of *E. coli* cells in each of the three microcosms systems at 18 °C, after inoculation in Levine culture medium, during the five days experiment

The slightly higher number of *E. coli* C.F.U. obtained on the Levin medium by comparison with LB culture medium could be probably explained by the superior nutritive composition of the Levin medium as compared with LB.

CONCLUSIONS

1. Within the five days period of this study there was a steadfast decrease of the density of live cells from each of the three microcosms. The decline suffered by the cell population was notable higher in the unfiltered sea water microcosm as compared with the results obtained in both microcosms with filtered sea water (0.22 µm or 0.45 µm).
2. Total cell count (SYBER- Green labelled cells) in the sterile microcosm (0.22 µm filtered sea water) was maintained constant during this study whereas in non-sterile microcosms (unfiltered or 0.45 µm filtered sea water) total cell count decreased. This suggest that n the sterile microcosm (0.22 µm- filtered sea water) physical disaggregation of cells does not occur, while for the other two microcosms nutritional competition with other microorganisms and microbial predation seems to play a (key) role in the reduction of total cell density of *E. coli* cells.
3. The density of colony forming units seems to be parallel both in sterile and 0.45 µm - filtered sea water microcosms but after 22 hours the decrease in C.F.U. is much higher in non-filtered microcosm, reaching extinction after 5 days both in LB and Levin media, suggesting the dramatic effect of predators on *E. coli*.

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