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THE VIABILITY OF *SALMONELLA ENTERICA* VAR. *POONA* IN FILTERED AND UNFILTERED SEA WATER MICROCOSMS AT DIFFERENT TEMPERATURES

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Abstract
This study focuses on the determination of the viability of Salmonella enterica var. poona sub-population submerged in indoor sea water microcosms for a period of five days. The usage of bought filtered and unfiltered sea water and two different temperatures (4°C and 37°C) aimed to establish the importance of biotic and abiotic parameters of the microcosms in the dynamic of the Salmonella enterica var. poona cells. The collected probes were analysed via fluorescent microscopy with respect to total cell density and the number of dead and the cultivability capacity of the bacteria cells which was assessed by CFU determination on selective culture media (Luria Bertani and Salmonella Shigella Agar). The results obtained in this study show that in this five days period the temperature of the sea water and the presence of concurrent bacteria and protozoa are contributing significantly to the decrease of viability and multiplication capacity of Salmonella enterica var. poona that undergone a hypo-osmotic shock after the passage in the indoor microcosms. Low temperature (4°C) and protozoa predation (unfiltered sea water) seemed to be the most harmful microcosm configuration regarding bacterial survivability leading to near annihilation of the cultivability capacity at the end of the five days studied time.

Keywords: Black Sea water, colony forming units, fluorescence, microcosm, Salmonella enterica var. Poona.

1. INTRODUCTION

Seawater contamination with potential pathogenic Enterobacteriaceae such as *Salmonella spp*., which poses great risk for both marine life and human health, has long been the concern and study objective for many researchers worldwide (Venkateswaran et al., 1989; Baudart et al., 2000; Catalao et al., 2000; Martinez-Urtaza et al., 2004; Baudart et al., 2011). Consequently, investigating the presence of *Salmonella spp*. in seawaters as an indicative of water pollution and analysing the natural abiotic and biotic parameters that determine its resistance and survival capacity, is and will be under continuous evaluation (Nabbut and Kurayiyyah, 1972; Rozen and Belkin, 2001; Lemarchand and Lebaron, 2003). Though, in comparison with the human digestive tract environment in which *Salmonella* species lead a predominantly host-associated lifestyle, the specific features of the Black Sea water, such as lower temperature and higher salinity, offers a rather hostile environment. Nevertheless, it can be considered a reservoirs due to shedding and polluted sewage (Goldrick and Barbara, 2003; Winfield et al., 2003). Therefore, this study aims to determine the dynamics of *Salmonella enterica* var. *poona* in sea water microcosms featuring filtered and unfiltered water at different temperatures, in order to establish the particularities of its viability and multiplication capacity in this specific condition. We consider that a study that takes

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into consideration the present stage of the Black Sea water specific characteristics related to a potential *Enterobacteriaceae* accidental multiplication is mandatory in order to prevent and better understand its dynamics.

2. MATERIALS AND METHODS

Strain and microcosms

The biological material was a TCS Biosciences NCTC 4840 certified *Salmonella enterica* var. *poona* strain that was grown on Salmonella Shigella Agar media (MacFaddin, 1985) at 37°C for 24 hours. The cells were aseptically washed two times in sterile phosphate buffer saline and introduced in the microcosms. Previously we tested this particular strain regarding the ability to form biofilm, as it is well known that *Salmonella spp*. has a tendency to form biofilms on glass surface materials (Vidal De Oliveira et al., 2014). Those experiments showed that for the tested strain of *Salmonella enterica* var. *poona* the biofilm formed on a glass surface in 72 hours was indeed exceedingly shallow and did not posed an impediment for the future cell counting in liquid phase.

Sea water was collected from the Constanta city Black Sea harbour waters, at a depth of 1 m. For this study, four microcosms were constructed in sterile glass bottles of 1 L each. For two of them the sea water was filtered thru 0.22 µm Millipore filters in order to exclude other bacteria and protozoa pre-existing in the sea water, thus ensuring a sterile environment. For the other two unfiltered water samples, was used an environmental configuration as close to the natural condition as possible, that provides bacterial competition and protozoa predation which is known to play a key role in the dynamics of potential pathogen bacteria such as *Salmonella spp*. (Barcina et al., 1992; Gonzales et al., 1992; Sherr, 2002).

In each of the four microcosms was added 1 mL of *Salmonella enterica* var. *poona* culture at OD660 =1.0. One 0.22 µm filtered sea water microcosm and one unfiltered sea water microcosm were kept for the duration of the study at 4°C in the dark. The similar pair was kept at 37°C, also in the dark. The tested samples were collected immediately after inoculation (time zero), and also at 1h, 3 h, 22 h, 46 h, 3 days, 4 days and 5 days.

Viability determination

Microscopy cell count

The samples were analysed by using fluorescence microscopy with respect to total cell densities, in order to determine the number of *Salmonella enterica* var. *poona* cells that are preserved viable, with functional plasma membrane, under the particular stress condition of the microcosms in this 5 days period. Therefore, each collected sample was stained with both SYBR Green and Ethidium homodimer. SYBR Green was used for its highly selectivity towards double stranded DNA, for labelling both live and dead cells (Zipper et al. 2004), while Ethidium homodimer, a membrane-impermeable fluorescent dye that does not cross intact/viable cell membranes, was used for labelling only dead/dying cells (Manini and Donovaro, 2006) (Figure 1 and Figure 2).

For the staining protocol, the cells from each sample were collected by centrifugation at 10.000 r.p.m., resuspended in sterile physiological saline and filtered through 0.22 µm polycarbonate Nucleopore filters with Millipore funnel, achieving a uniform distribution over the filtration surface. The readings were performed with an 63x objective, on an average of 20 microscopic fields, counting 300-600 cells for each probe and the cell density was determined following the Manini and Donovaro formula (2006):

Cell density/mL = { [3,14 x (75000x75000)]:(90 x 106) }x number of counted cell per field/1,5

First it was determined the total cell number of *Salmonella enterica* var. *poona* (SYBER Green stained) for each sample. The same calculus was made with respect to dead cells density (Ethidium

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homodimer). By subtracting the number of dead cells from the total cell count for each sample it was acquired the number of live cells.

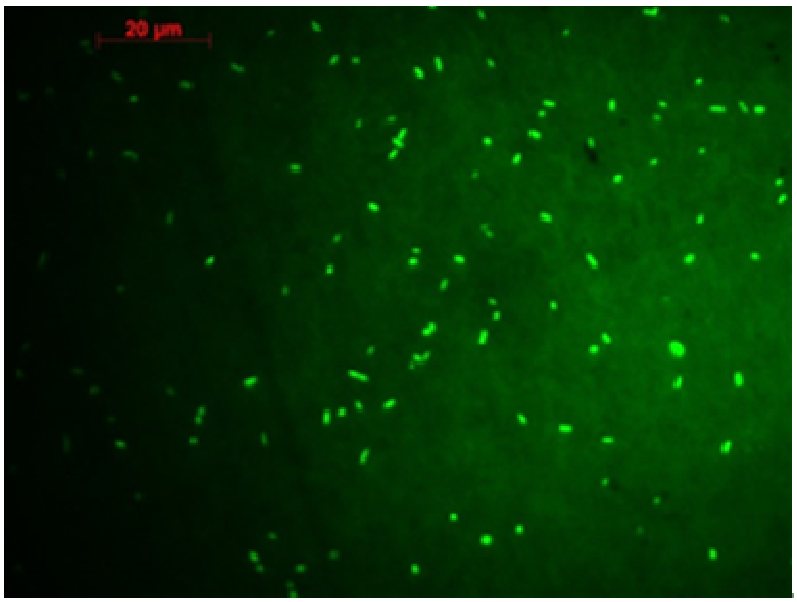

Figure 1. Salmonella enterica var. poona cells stained with SYBR Green (Ob. 63x)

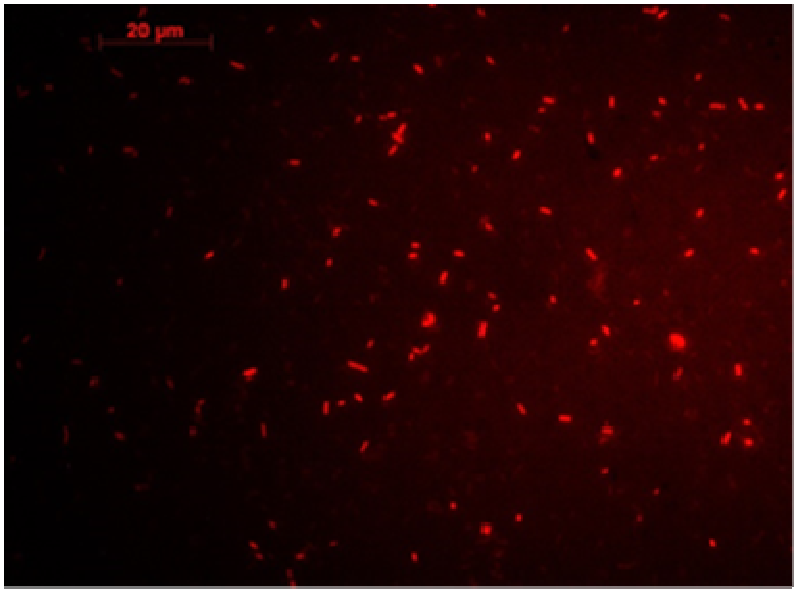

Figure 2. Salmonella enterica var. poona cells stained with Ethidium homodimer (Ob. 63x)

Colony Forming Units determination

Besides the microscopy quantification, this study aimed to determine the viability of *Salmonella enterica* var. *poona* under the sea water microcosms stress condition. Therefore, it was analyzed the ability of cells to grow and multiply when transferred back from the microcosms to selective culture media. Fresh subsamples of 10 µl each were collected from all microcosms and inoculated by the droplet method (Neblett, 1976; Hoben and Somasegaran, 1982) on both Luria Bertani (LB) and Salmonella Shigella (SS) Agar. Two different culture media were used in order to avoid any

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bacterial interferences form the unfiltered sea water microcosms and also for a better CFU quantification. Salmonella Shigella (SS) Agar is a moderately selective and differential medium for the isolation, cultivation and differentiation of *Salmonella spp*. in which the bacterial colonies appear colourless with black centres (MacFaddin, 1985) (Fig. 3). The plates were incubated at 37°C for 24 hours.

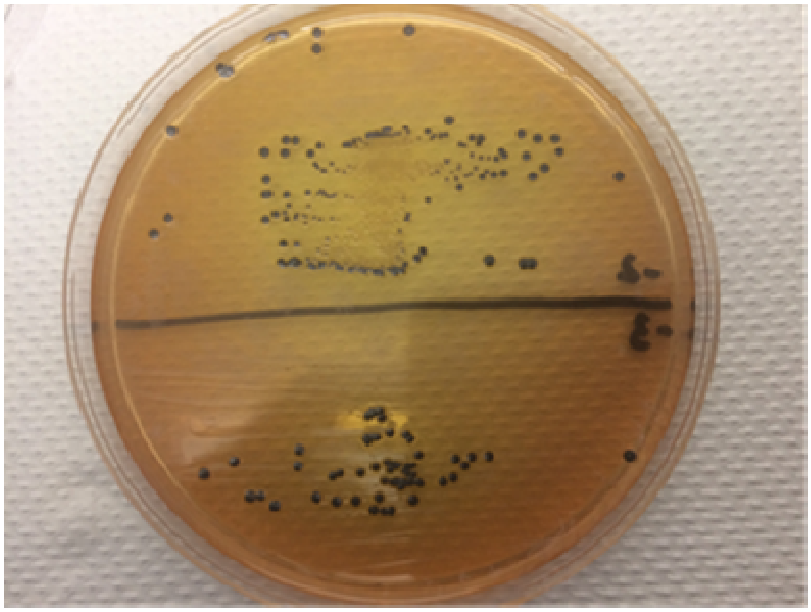

Figure 3. Salmonella enterica var. poona CFU on SS Agar after 24 hours at 37°C

3. RESULTS AND DISCUSSIONS

The total cell count (SYBR Green labelled cells) practically remained unchanged in each microcosms (results not shown) suggesting that for this period of time cell disintegration and biofilm formation is highly unlikely. However, when it comes to alive cells (SYBR Green labelled cells minus Ethidium homodimer labelled cells) in the first 3 hours (Figure 4), their density in all four microcosms, with both filtered and unfiltered sea water, remained constant, regardless of the environment temperature.

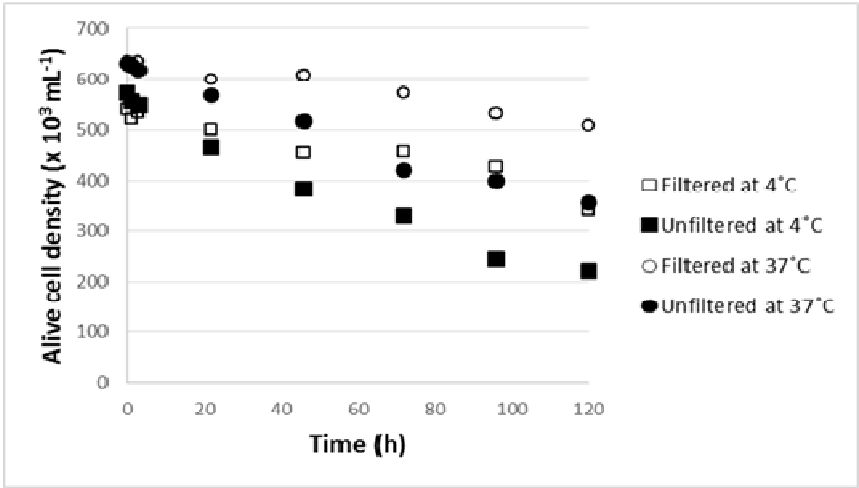

Figure 4. Time evolution of alive Salmonella enterica var. poona cells densities (total cell-SYBR Green - minus dead cells-Ethidium homodimer) in filtered and unfiltered sea water, at 4°C and 37°C

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However, after 22 hours there was a drastic decrease of live cells at 4°C as compared with the cells kept at 37°C. *Salmonella enterica* var. *poona* is a potential pathogen faecal bacteria that usually grows and amplifies in the digestive tract of warm blooded mammals, at 37°C, thus the cells from the microcosm with sea water featuring the same temperature excluded the thermal shock, therefore their viability is not so severely affected. These results are in agreement with those reported in literature for *Salmonella spp*. (Haley et al., 2009; Gülşen, 2012). The fact that in the first three hours there are no differences between microcosms with respect to the number of live cells is merely attributed to the short time span, as it has been formerly advocated in other studies (Moriñigo et al. 1990).

With respect to *Salmonella enterica* var. *poona* multiplication capacity, one can observe a decrease in the number of CFU in all four microcosms, during the five days period (Figure 5 and Figure 6). Although there are studies that confirm a loss of cultivability of *Salmonella spp*. cells in the first 48 hours (Roszak et al., 1984), it can be presumed that the osmotic, saline and nutritional stresses are the main contributing factors (Troussellier et al., 1998; Bordalo, 2008). The major difference attributed to predation, regarding the *Salmonella enterica* var. *poona* population from the unfiltered sea water microcosms, that can be observed in the cells quantification by microscopy, its manifested also in colony forming capacity. This decrease has been highly noted from the third day on, leading to nearly total annihilation of cells that are able to grow and multiply by day five (Figure 6).

On the other hand, can be argued that the combination between the osmotic and saline shock with the nutritional competition and protozoa predation stress can lead to the appearance of a nonculturable state of the *Salmonella enterica* var. *poona* cells. The state of viable but nonculturable cells has already been stated for faecal enterococcus bacteria such as *Salmonella spp*. that suffer increasing environmental stress in which they maintain their viability but are not able to divide by colony forming units even when suspended in nutritional, specific culture media (Roszak and Colwell, 1987; Troussellier et al., 1998).

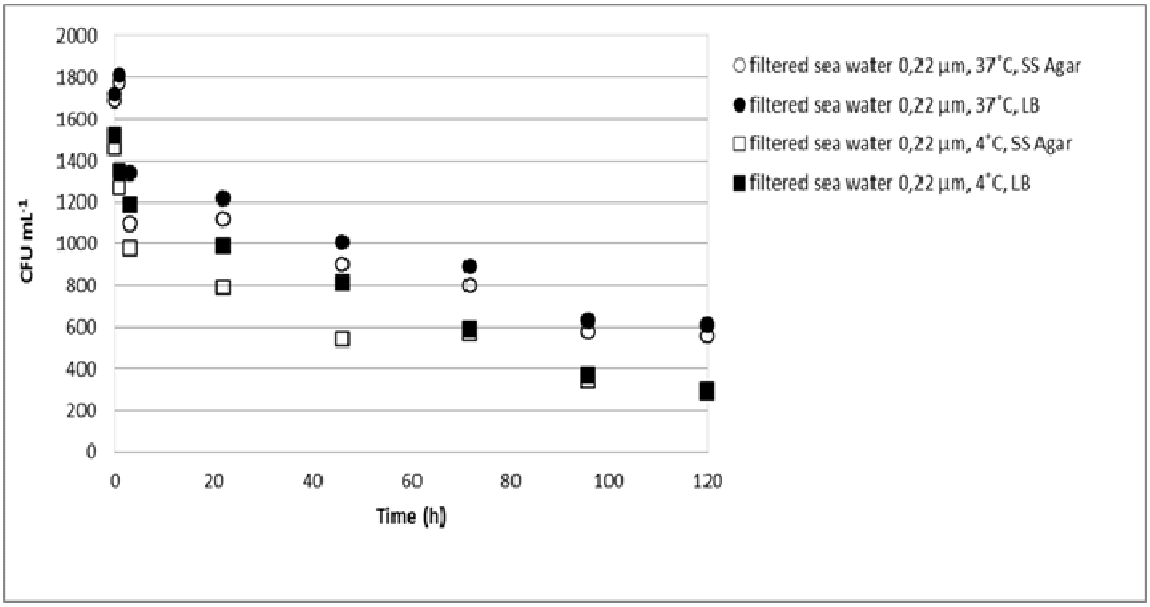

Figure 5. Number of Salmonella enterica var. poona cells able to grow and multiply (CFU) on SS Agar and LB media after sample harvesting from filtered sea water microcosms kept at 4°C and 37°C

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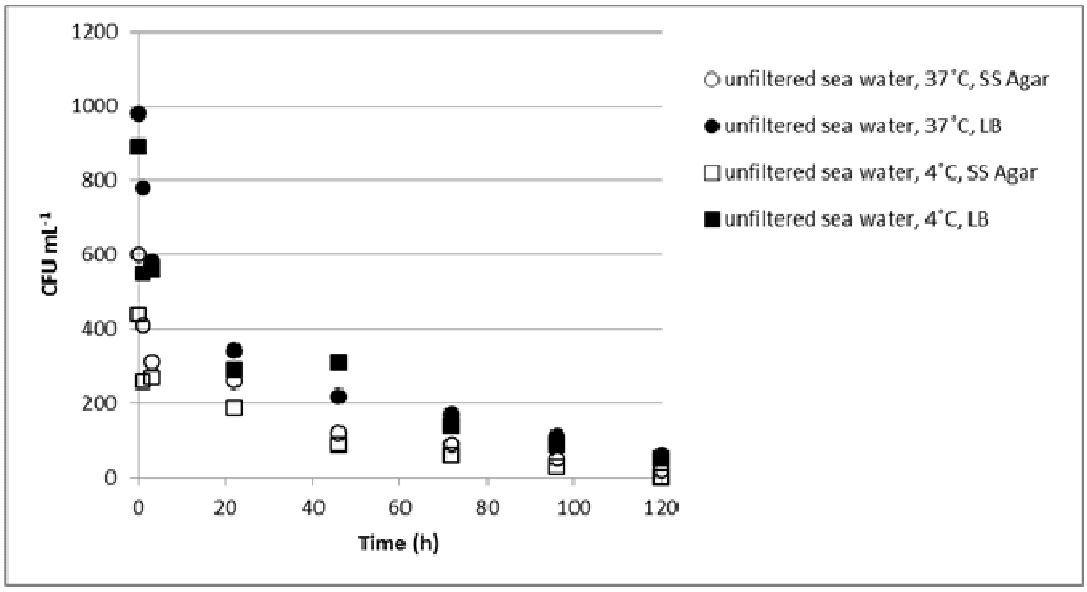

Figure 6. Number of Salmonella enterica var. poona cells able to grow and multiply (CFU) on SS Agar and LB media after sample harvesting from unfiltered sea water microcosms kept at 4°C and 37°C

These results are in agreement with other reports, as it has been previously established, predation mostly by protozoa such as dinoflagellates and competitive bacteria contributing significantly to the death of enterococcus bacteria like *Salmonella spp*. (Nabbut and Kurayiyyah, 1972; Schnepf and Elbrächter, 1992; Mezrioui et al. 1995; Rozen and Belkin, 2001).
Furthermore, the higher number of CFU grown on LB media in contrast with the number of CFU grown on SS Agar media suggest that, at least a part of the *Salmonella enterica* var. *poona* population from the microcosms, is losing its cultivability on selective media while maintaining it on a non-selective media, as stated in other studies (Moriñigo et al, 1990).

4. CONCLUSIONS

Sea water temperature is an essential parameter for the viability of *Salmonella enterica* var. *poona* introduced in the microcosms - the density of alive cells and of that of CFU being higher at 37°C than at 4°C.
In the presence of bacterivores and other bacteria (microcosm with non-filtered sea water) the decrease in the cells viability is very sharp as compared with filtered microcosms, the density of live cells and of that of CFU being very low, or even absent.

5. ACKNOWLEDGEMENTS

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