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Occurrence and Survival of Pathogenic Microorganisms in Irrigation Water

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1. Introduction

The consumption per capita of fresh vegetables has increased in the last years in the USA and other countries, which has contributed to the increase of gastroenteritis outbreaks attributed to contaminated fruits and vegetables. Fresh produce can incorporate pathogenic microorganisms thru the process of irrigation, harvesting, postharvest processing and distribution. Most microorganisms use irrigation water and/or soil as a vehicle of transport (Beuchat, 1995; Bhagwat, 2003). Untreated water is most likely to transmit several microorganisms, which may include pathogenic strains of *Escherichia coli*, *Salmonella*, *Listeria*, protozoa and viruses (Díaz *et al*., 1999). Studies in different countries indicate that the use of untreated water for irrigation of vegetables is the practice most related to fresh produce safety issues (Monge*et al*., 1996; Díaz *et al*., 1999; Tyrrel y Quinton, 2003).

Surface water may pose a risk of contamination if its source is unknown. Water is used for multiple issues in diverse agricultural activities including application of fertilizers, washing and disinfecting produce. Therefore, water has to meet the chemical and microbiological requirements before its use (Siller *et al*., 2002). The quality of water is based on the amount of indicator microorganisms. The major source of coliform contamination when vegetables are grown is probably the irrigation water (Okafo*et al*., 2003). There are critical factors that need to be monitored to ensure safe water supply. All water sources must be examined periodically for microbiological determination, the results must be recorded and existing problems corrected, for example bathing and grazing animals nearby water resources which should be prohibited to prevent fecal contamination and reduce risks to human health from consuming contaminated fresh produce.

Frequently, contamination is associated with the application of irrigation water and the type of crop. Studies have proved that flooding irrigation represents the greatest possibility of contamination if it's used on produce having direct contact with the soil while the sprinkler irrigation technique provides a rapid means to contaminate the product if the water is contaminated; On the other hand, the drip irrigation technique has represented the lowest risk of contamination of produce (Siller-Cepeda *et al*., 2009). Lettuce, radishes, carrots are vegetables most likely to become contaminated due to their direct contact with soil and water which can possibly contain bacteria (Okafo*et al*., 2003). It is well recognized that fecal indicator bacteria may be transported and be a source of contamination of water sources (Tyrrel and Quinton, 2003).

Fecal and total coliforms are considered the index parameter of pathogenic microorganisms, but recently this role has been questioned because of its ability to grow in various water sources (Gleeson, 1997; Banning *et al*., 2002).

Since its first isolation and description in 1982, *E. coli* O157:H7 has been associated with numerous outbreaks in the US and some other countries in the world (Bitton, 1994). From the foodborne outbreaks occurred in Japan, the 1996 radish outbreak is among the most deadly with thousands of people ill and 11 school children deaths. Scotland also suffers from an outbreak due to the consumption of undercooked meat that caused the deaths of 16 elderly (Loaharanu, 2001).

Listeria monocytogenes is a Gram positive organism found in the environment and cause disease in animals and humans. The host susceptibility is important for the development of the disease having a primary target pregnant woman, immunocompromised persons, elderly and newborns. One of the most important properties is the intrinsic resistance to the environment and its growth at broad ranges of pH (4.5 – 9), temperature of 0 to 45°C and Aw (0.92) (Copes *et al*., 2000). This bacterium can survive for long periods in soil and crops; it does not lose its virulence during the period on the soil or the environment becoming a health hazard if ingested thru contaminated fresh produce. The route of transmission of Listeria is by the ingestion of contaminated food and water (Al-Ghazali and Al-Azawi, 1990).

Salmonella include more than 2500 pathogenic serotypes. It is ubiquitous in the environment and is commonly found in water, food and other materials. This pathogen colonizes the intestinal tract of humans, animals, birds and insects and is transmitted to the irrigation water, drinking water, mild and other raw foods by fecal contamination (Abushelabi*et al*., 2003). It is also commonly found in reusable water and contaminate by runoff of rivers, discharges into the sea and other sources of agricultural water (Lemarchand and Philippe, 2002).

Microbiological contamination of pathogenic bacteria in vegetables becomes more important when considering that survival time may be prolonged for weeks or months, particularly when they are protected from desiccation and direct sunlight, as in lettuce, cabbage and radish. Studies have shown that pathogens inoculated into farmland or irrigation water can survive for up to two months, a period sufficient to reach the consumer (Monge*et al*., 1996).

Survival is defined as the ability to maintain viability with adverse circumstances. Bacteria in water respond to different physical and chemical variables including low or high concentration of dissolved oxygen, redox potential and pH (Roszak and Colwell, 1989). When bacteria get in contact with the surface of vegetables can initiate a process of adhesion and subsequent colonization under favorable conditions. Some cells produce extracellular polymers that lead to the formation of biofilms. This process is usually accompanied by an increased tolerance to the effect of drying and the lethal action of ultraviolet light. Concurrently microorganisms protect themselves staying at sites such as cracks, depressions, stomata, lenticels and trichomes that are part of the structure of vegetation (Fernández, 2001).

The existing national standards establish the limits of microbial contamination only for wastewater. There are no rules governing the use of irrigation water at the national or international level. The amount of microorganisms varies considerably in agricultural water and wastewater, this type of water is ruled by the NOM-003-ECOL-1997 that establishes the maximum contaminant limits for treated wastewater to be reused. The FAO/WHO 1989, set as the maximum level of 10^3 fecal coliforms/100 mL (Monge *et al*., 1996).

One of the problems facing growers of the Valley of Culiacan is the shortage of water due to lack of rain, the indiscriminate use of the agricultural sector and the growing population. The valley of Culiacan is located within 010 irrigation districts consisting of 8 modules of irrigation, of importance to this study are II-1, II-2 and IV-1, which supply water to the main producers of agricultural fresh produce in our state (Table 1).

Fresh Produce Crops	**Irrigation Districts**		
	Module II-2	Module III-3	Module IV-1
Tomato	✓	✓	✓
Cucumber	✓	✓	✓
Bell pepper	✓	✓	✓
Eggplant	✓	✓	✓
Squash	✓	✓	
Scallion	✓	✓	
Swiss chard	✓		
Onion			✓
Green beans	✓		✓

Table 1. Fresh produce crops irrigated with superficial water through the irrigation modules in the Culiacan Valley.

There are no studies on the presence and survival of these bacteria in water used to irrigate vegetables in the valley of Culiacan. Because of this reason it was proposed to determine the incidence of *Escherichia coli*, *Salmonella* spp and *Listeria* spp in canal water for agricultural use and to determine its survival exposed to different physicochemical parameters in laboratory conditions.

2. Methods

The study was divided in *Field Study* and *Lab Study*:

2.1 Field study

It was performed a correlational descriptive study to identify bacteria and their survival by using a three factor completely randomized repeated measurements. Seventy samples of water from canals in the Culiacan Valley where taken in two stages, the first one from February to May 2003 (fresh produce production ending, rainy season) and the second one from November 2003 to February 2004 (fresh produce production pick, drought season). Sampling points were determined randomly according to the area of greatest fresh produce production in the Culiacan Valley, Sinaloa, specifically in the irrigation modules II-2, II-3 and IV-1 including crops of tomato, cucumber, squash, eggplant among others (Table 1). Sinaloa state is located at the northwest of Mexico (27° 7´-22° 20´N, 105° 22´-109-109° 30´O) (Figure 1) with a population of 2,767,761 people (INEGI, 2011).

Collection of samples

The samples of water were collected in sterile 1-L bottles, properly labeled and sealed. Transportation to the laboratory was conducted under refrigeration (4-6°C) for the

microbiological analysis. The study analyzed a total of 70 samples, where the parameters turbidity, temperature and pH were measured in the field.

Microbiological analysis

Fecal coliforms were determined by the membrane filtration technique (APHA, 2001). One and 10 mL of water samples were filtered through a membrane of 0.45 μm and plated on m-FC agar (Difco) and incubated 24 h at 45°C. For the biochemical determination of *E. coli* the API 20E system was used (Biomeriux Vitek, Hazelwood, MO) by placing a positive coliform colony in 5 mL of 0.85% sodium chloride saline solution to obtain a bacterial suspension, which was added in the gallery microtubes and incubated at 37°C during 24 h. Gallery reading was based on the API 20E table. For the isolation of *Salmonella* spp and *Listeria* spp enrichment broths and selective agars were used as describe by APHA (2001).

2.2 Lab study

The second phase of the study consisted in an *in-lab study* performing bacterial survival analysis at different temperature, turbidity and pH. For this, positive samples of *Escherichia coli* and *Salmonella* spp obtained from the *Field Study* and confirmed by API 20E were used. *Listeria* spp was obtained from the State Public Health Laboratory.

Bacterial Regeneration

A colony of the bacterial strains was isolated and transferred separately in 5 mL of soy broth trypticasein (TSB) which was incubated for 24 at 37°C. Once the incubation time elapsed, 1 mL of this bacterial suspension was taken and added in an Erlenmeyer flask containing 50 mL of TSB, being incubated again for 24 h at 37°C. The cultured broth was placed in tubes (Beckman J2-MI) and centrifuged at 10,000 rpm, 4°C for 11 min. The precipitate obtained was re-suspended twice with 15 mL of phosphate buffer (KH_2PO_4 pH 7.2) stirring vigorously with a vortex mixer (Scientific Products) to obtain a homogeneous solution. This process was repeated twice (Abbaszadegan *et al*., 1997). Finally, the pellet obtained was suspended in 300 mL of buffer reaching a concentration of $1x10^8$ colony forming units per milliliter (CFU/mL).

Survival Evaluation

Survival evaluation followed the methodology described by Johnson et al. (1997), with some modifications described below. A liter of canal water was inoculated with 30 mL of the three regenerated bacteria reaching concentrations of $1x10^7$ CFU/mL and different temperature (15, 35, 40°C) and turbidity (2, 20, 50 Nephelometric Units, NTU) were adjusted. These mixtures were exposed to the environment with constant stirring for 72 h. Aliquots were taken at different exposure times (0, 3, 12, 24, and 48). Dilutions were performed and 0.1 mL was stricken onto m-FC agar (Difco) for the identification of *E. coli*, Hektoen agar (Difco) for *Salmonella* and Palcam agar (Difco) for *Listeria* and finally incubated at 35 ± 0.5°C for 24 h. Each treatment was performed in duplicate.

2.3 Statistical analysis

The analysis for the quantification of coliforms was average in the three irrigation modules, while for the survival analysis a three factors design completely randomized with repeated measures over time was performed with MINITAB program with a significance level of $p > 0.05$.

3. Results

3.1 Field study

During the *first stage* there was a low rainfall contribution. The highest fecal coliforms average was detected in the irrigation module II-2 with more than 40000 CFU/100 mL (Table 2). The temperature remained constant during the sampling period ranging between 33 and 35°C, behaving similarly than the pH with a value of 7.1 while total chlorine was registered with an average of 0.9 ppm. The difference was observed in turbidity registering levels higher than 50 NTU (Table 2).

Parameter	Module II-2	Module III-3	Module IV-1
Fecal coliforms (CFU/100 mL)	40000	23000	28000
Temperature (°C)	35	33.5	34.5
pH	7.13	7.16	7.13
Turbidity (NTU)	48.43	56.5	17.08
Total chlorine (mg/L)	0.11	0.08	0.08

Table 2. Microbiological and Physicochemical quality of water during the first stage February-May, 2003.

During the *second stage* there was a rainfall contribution and it is called the rainy season. In this *stage* of sampling it was observed fewer fecal coliforms with respect to the first stage, presenting the highest numbers the module III-3 with more than 7500 CFU/100 mL, similar levels to those of the IV-1 module with 7000 CFU/100 mL. The temperature was kept within a range not exceeding 22°C and the presence of total chlorine less than 0.2 ppm (Table 3).The temperature was kept within a range no greater than 21.2 °C and the presence of total chlorine was less than 0.2 parts per million.

Parameters	Module II-2	Module III-3	Module IV-1
Fecal coliforms (CFU/100 mL)	4000	7500	7000
Temperature (°C)	21.2	20.3	20.6
pH	7.08	7.5	7.2
Turbidity (NTU)	30	48	49
Total chlorine (mg/L)	0.07	0.15	0.13

Table 3. Microbiological and Physicochemical quality of water during the second stage November, 2003 – February, 2004.

In table 4 can be observed that the bacterium found in greater proportions during the *first stage* was *E. coli* in the three irrigation modules, accumulating a total of 36 positive samples out of 40 (90%). The presence of *Salmonella* is noted but to a lesser extent with a total of 8 positive samples (20%). In the other hand the presence of *Listeria* spp was not detected.

Irrigation districts	*E. coli*	*Salmonella* spp	*Listeria* spp
II-2	12	2	0
IV-1	10	2	0
III-3	14	4	0
Total	36	8	0

Table 4. Number of positive samples for *E. coli,Salmonella* and *Listeria* during the first stage february-May, 2003.

Results of the presence of pathogenic bacteria during the *second stage* show *Escherichia coli* with a highest incidence of 63.3 % positive samples, while *Salmonella* spp. represented only a 6.6 %. Finally, *Listeria* spp was not detected as in the *first stage*.

Irrigation districts	*E. coli*	*Salmonella* spp	*Listeria* spp
II-2	5	1	0
IV-1	8	1	0
III-3	6	0	0
Total	19	2	0

Table 5. Number of positive samples for *E. coli, Salmonella* and *Listeria* during the second stage November, 2003 to February, 2004.

It has been shown that the frequent contamination of soil is due to the application of contaminated water or animal and humans feces. However, microorganisms have strategies that enable them to face the adverse environmental factors and allow pathogens to remain viable in soil for two months or more especially in shaded moist areas (Solomon *et al*., 2002; Monge *et al*., 1996). This could explain at least partially fecal contamination during the rainy season in those vegetables whose edible portion is in direct contact with the ground (Cifuentes *et al*., 1994, Monge *et al*., 1996). Tyrrel y Quinton (2003) mention that the transport of coliform in water is mediated by the density and turbidity of the water, which means when there are continuous rainfall the volume of water bodies increases, causing a dilution in the total coliforms originally present which coincides with the present investigation founding decreased concentrations in the second stage sampled in different modules. In spite of the low concentrations, epidemiological studies have shown that there is a risk of gastroenteritis, when people are indirect contact with 20 to 35 CFU /100 mL fecal population (Polo et al., 1998). In the present study there were found higher levels of fecal contamination in canal which represent a risk of contamination for vegetables that come into contact with it and furthermore for consumers.

Polo *et al.* (1998) reported absence of *Salmonella* in the presence of fecal coliforms in river water and can be explained by interference of aquatic micro biosphere, moreover the authors mention the possibility of mutation that has bacteria in order to be found in the environment as a viable but not cultivable. Okafo *et al.* (2003) found a relationship between the incidence of pathogenic bacteria of 1 *Salmonella* per 45 total coliforms and 13 fecal coliform in water samples. However, the most conclusive result will always be to identify pathogenic bacteria rather than index organisms. Table 4 shows a higher proportion of

Escherichia coli in general making a total of 19 positive samples out of 30. *Salmonella* spp was isolated in a lower proportion (2 positive samples) while *Listeria* was not detected in any of the samples. *Listeria* compared with the Gram negative bacteria shows a survival decrement in aquatic environments, its characteristics as Gram positive bacteria confers the cell with poor resistance to those conditions (Liao and Shollenberger, 2003). In this sense, Monfort *et al.* (2000) refers to *Listeria* as a bacterium resistant in food, refrigerated temperature and low oxygen conditions, however they also mark its low survival in aquatic environments at different stress conditions.

3.2 Lab study

Escherichia coli survival

The survival profile shown in all treatments was descendent, but different between factors. At temperature of 15°C with a 50 NTU turbidity reaching the maximum time of survival registered 4.3 $\log_{10}$ in contrast at temperature of 35°C with 50 NTU turbidity which reached a concentration of 3.4 $\log_{10}$ at the end of the experiment, the difference is greater at 40°C and at 50 UNT reaching a concentration of 2.7 $\log_{10}$. This indicates that the high turbidity and lower temperature increase the survival time for *Escherichia coli* under these conditions.

The temperature factor was analyzed showing 35°C as the best temperature for the *E. coli* survival (Figure 1A). When analyzing this factor using the Tukey test ($p<0.05$) showed statistical differences. Numbers under the same line are not statistical different.

15 35 40

According to the principal effects graphic the *Escherichia coli* survival order based on turbidity was 50 UNT > 20 UNT > 2 UNT (Figure 1B). Statistical differences were observed when the Tukey test was performed ($p<0.05$).

50 20 2

Along time bacterial concentration presented a considerably decrement (Figure 1C). Four different contact times where statistical different ($p<0.05$).

48 24 12 0

E. coli presented a longer survival time when exposed to 50 NTU finding a correlation between turbidity and survival time. In other words, the increased turbidity improved survival. When organic matter is present in aquatic environments there is a protective effect of bacteria on organic matter, against the direct rays of the sun (radiation, UV light), which coincides with the results obtained by Mezriqui *et al*. (1995) who conducted a study with *E. coli* and *S*. Typhimurium in sea water finding that water with higher turbidity contained higher concentrations of bacteria and survived for longer period of time. A similar study in which the survival time was measured at different total suspended solids (TSS) concentrations and different solar radiation, found significant differences in solar radiation with respect to TSS, obtaining that a higher radiation and lower TSS further reducing the survival in stagnant water, finding a relationship between increased bacterial survival and greater amount organic matter with respect to time.

In aquatic environments nutrient limitation is a major stressor for the cells. Gram-negative, particularly *E. coli* and *Salmonella* generally increase resistance to environmental factors at the stationary phase. *Escherichia coli* have the ability to survive in aquatic environments even at low temperatures (Rice and Johnson, 2000).

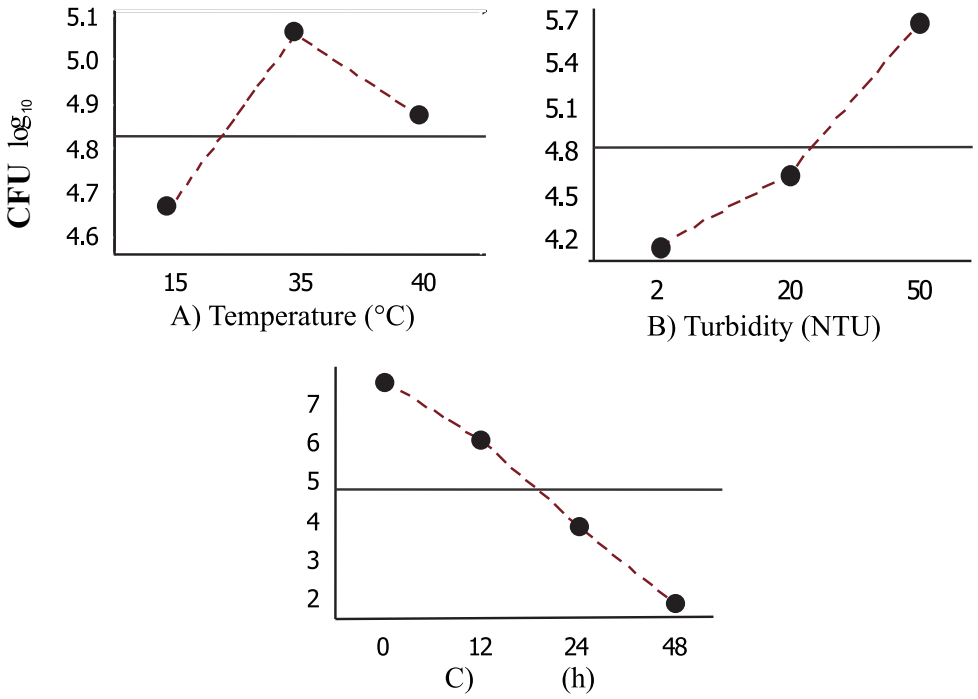

Fig. 1. Principal effects graphic of the three abiotic factors on *Escherichia coli* survival. A) Different levels of the temperature factor, B) Different levels of the turbidity factor, C) Different levels of the time factor.

Salmonella sp Survival

During the 48 h experiment *Salmonella* sp showed a steady decline, but different between the levels of temperature. The temperature of 15°C showed the highest survival over time getting the highest growth at 12 h (6.3 $\log_{10}$) and decreasing its concentration at 24 h to 3.9 $\log_{10}$, to finally registering a concentration of 1.5 $\log_{10}$ at 48 h (Figure 2C).

The survival displayed by *Salmonella* sp was greater at 50 NTU in the course of time (Figure 2A). The initial concentration was 7.4 $\log_{10}$, which was then descending considerably to reach 6.2 $\log_{10}$ after 12h of exposure of abiotic factors. In this sense at 24 h presented a concentration of 4.5 $\log_{10}$ to finally obtain 2.5 $\log_{10}$ at the end of the experiment (48h).The turbidity of 2 and 20 showed a lower bacterial concentration in the course of time detecting levels up to1 $\log_{10}$ after 48 h.

Salmonella sp survival was high when combining 15°C and 50 NTU. The condition of survival of bacteria over time was enhanced by the effect of high turbidity and low temperature.

ANOVA analysis shows that most variability between treatments was due to the effect of turbidity, temperature and time, which were statistically significant ($p < 0.05$).
According to the main effects plot bacterial survival between different turbidities showed differences and by Tukey test ($p<0.05$) differences were significant (Figure 2A).

2 20 50

For temperature significantly differences (Figure 2B) were detected by the Tukey test ($p<0.05$).

15 35 40

Regarding the time factor represented a cause of decreasing variability in the course of the experiment on *Salmonella* sp survival (Figure 2C).

48 24 12 0

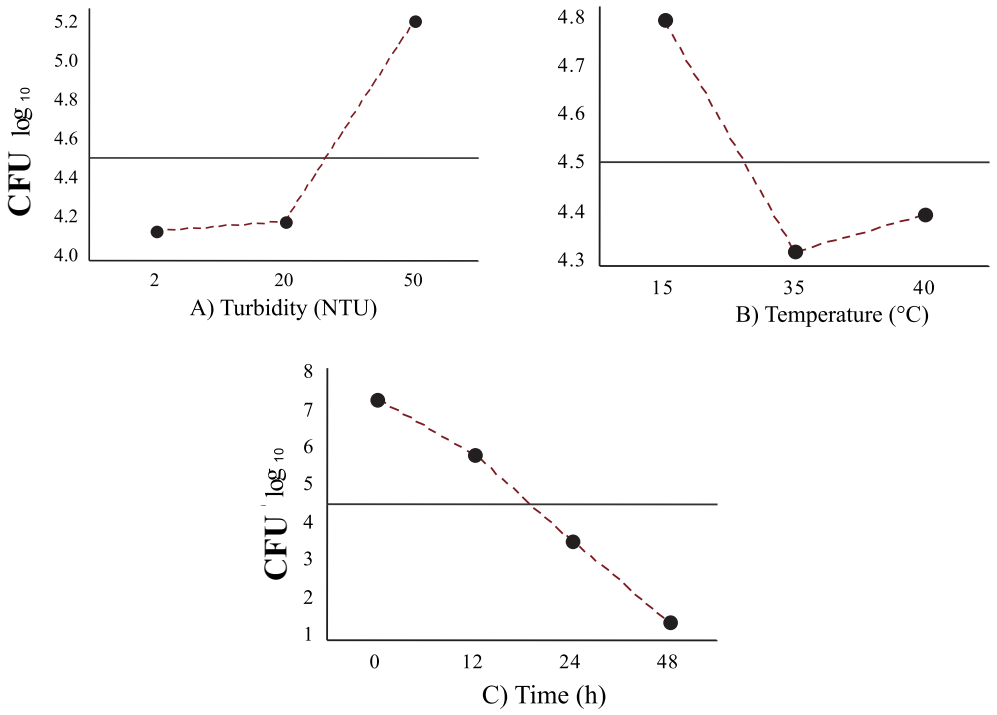

Fig. 2. Principal effects of the three abiotic factors on *Salmonella* survival. A) Different levels of the turbidity factor, B) Different levels of the temperature factor, C) Different levels of the time factor.

Lemarchand and Lebaron (2002) mentioned that in the stationary phase, *Salmonella* spp serotypes present in the environment may be constantly changing and developing resistance to a variety of environmental factors, therefore the results of this research show a positive correlation in the survival of *Salmonella* spp with respect to survival time at 15°C being this temperature as the most suitable to sustain *Salmonella* survival.

Other studies indicate that *Salmonella* sp has different survival mechanisms, such as decreasing metabolic activity and being viable but not cultivable in the environment, using genetic changes in its structure, likewise different defense mechanisms for protection that are activated in stationary phase such as the production of protective enzymes in the cell wall have been reported (Jonge *et al*., 2003, Santo Domingo *et al*., 2000).

In the present study it was observed an enhanced survival of *Salmonella* in high turbidity environment. These results are consistent with those reported by Mezriqui et al. (1995) whom reported the protective effect of high turbidity when UV light action in high turbidity at the lethal action of UV light from sunlight is affected by the high turbidity found in water used for the experiment.

Listeria sp Survival

Survival of *Listeria* sp was higher at 15°C (Figure 3). However, the concentrations found in temperatures of 35 and 40°C with 4.1 and 4.0 log $_{10}$, respectively were not statistically different.

The temperature of 15°C showed higher *Listeria* survival at all three levels of turbidity. The bacterial concentrations found at 50 NTU ranged from 5.2 to 5.4 $\log_{10}$, whereas at temperature of 35 to 40°C bacterial populations decreased significantly. The turbidity of 2 NTU presented a decrease in bacterial concentrations (3.5 $\log_{10}$). In general, the temperature of 15°C and 50 NTU presented a higher advantage improving *Listeria* survival.

ANOVA analysis showed that most variability between treatments was due to the effect of all the factors presenting statistical significance ($p < 0.05$).

The results for the temperature factor for *Listeria* sp revealed significant differences. Differences were found at 15°C, which had concentrations of 5.1 $\log_{10}$, compared to 35°C and 40°C with a concentration of 4 $\log_{10}$ and 4.1 $\log_{10}$, respectively.

15 35 40

According to the main effects plot bacterial survival between different turbidities showed differences and by the analysis of the Tukey test ($p<0.05$) differences were observed with respect to the highest turbidity.

2 20 50

The factor time had a decreasing variability effect in the course of the experiment on *Listeria* sp survival. The Tukey test ($p<0.05$) revealed significant differences.

48 24 12 0

It was evident that *Listeria* sp could survive longer periods of time at 15° C than 35 and 40°C, since this bacterium develops at low temperatures with an optimum growing temperature of 4°C (Murray et al., 1997). Monfort et al. (2000) conducted a comparative survival study of *Listeria inocua* and *Salmonella* Panama in pond water for agricultural use,

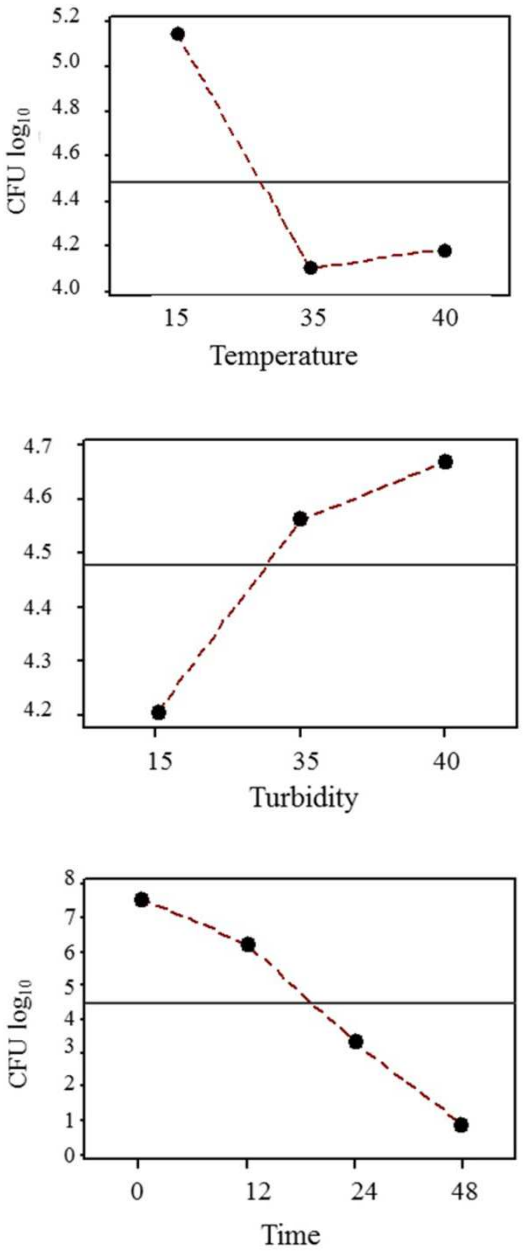

Fig. 3. Principal effects of the three abiotic factors on *Listeria* sp survival. A) Different levels of the temperature factor, B) Different levels of the turbidity factor, C) Different levels of the time factor.

where *Listeria* survival was favored at 4°C and in contrast to elevated temperatures (18°C), this data is consistent with the found in the present investigation, obtaining final concentration of *Listeria* sp at 15°C in 48 hours of 5.1 $\log_{10}$ while at high temperatures of 35 and 40°C the concentration was 4.0 $\log_{10}$. These results are similar to those reported by several studies that state that this bacterium has a higher survival at low temperatures, as optimal grow this 4°C (Szabo et al., 2003; Garrec et al., 2003).

4. Conclusions

When water comes into contact with fresh produce, the possibility of contamination from this source depends on its quality and origin. The risk of microbial contamination of water used in irrigation and processing activities should be reduced.

The results show evidence that *Salmonella* and *Escherichia coli* are present in irrigation water and can represent a potential risk for human health when they get in direct contact with edible vegetables. Therefore, control strategies must be implemented.

The survival of these bacteria was similar for the three different factors, but low temperature and high turbidity seem to represent the most suitable conditions for bacterial survival. However *Listeria* sp survival in canal water is limited.

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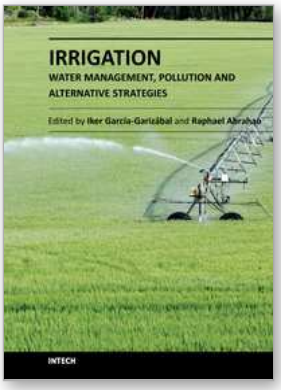

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Irrigated agriculture is the most significant user of fresh water in the world and, due to the large area occupied, is one of the major pollution sources for the water resources. This book comprises 12 chapters that cover different issues and problematics of irrigated agriculture: from water use in different irrigated systems to pollution generated by irrigated agriculture. Moreover, the book also includes chapters that deal with new possibilities of improving irrigation techniques through the reuse of drainage water and wastewater, helping to reduce freshwater extractions. A wide range of issues is herein presented, related to the evaluation of irrigated agriculture impacts and management practices to reduce these impacts on the environment.

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