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Palacios M.P., Lupiola P., Fernandez-Vera F., Mendoza V., Tejedor M.T.

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SALMONELLA TRANSPORT AND PERSISTENCE IN SOIL AND PLANT IRRIGATED WITH ARTIFICIALLY INOCULATED RECLAIMED WATER: CLIMATIC EFFECTS

M.P. Palacios *, P. Lupiola ** F. Fernandez-Vera *, V. Mendoza * and M.T. Tejedor ****
*Agronomía Fac Veterinaria. Universidad de Las Palmas de Gran Canaria mpalacios@dpat.ulpgc.es
**Microbiología. Fac Veterinaria. ULPGC. 35416 Autovia Las Palmas Arucas km 6.5
***Granja Agrícola Experimental Cabildo de Gran Canaria 35416 Autovia Las Palmas Arucas km 6.5

SUMMARY - The acceptability of reclaimed water to replace other resources for irrigation is dependent on whether the health risk and environmental impacts entailed are acceptable or not. Secondary effluent chlorination is extended but there are incidences in which *Salmonella* survive contaminating reclaimed water ponds. Specific site conditions influence pathogens existence and persistence. The aim of the experiments was to increase the scientific information on the climatic and soil effects on pathogens transport and survive under agricultural conditions.
Results from three experiments are presented here. Five pots were irrigated using a secondary effluent artificially inoculated with *Salmonella.* To study the effect of competence with soil bacteria, plates with sterilized soil were also tested. Radiation effect was also studied by blocking UV light. Stems and leaves, three different soil depths (surface, 0.15 and 0.45 m) and plates were sampled. As result of the radiation effect, less bacterial counts on plant were obtained during the day. But during the night *Salmonella* was able to re-grow, depending on the nocturnal temperatures. After 3 weeks in the field, Salmonella was still detected in plant samples. Radiation caused *Salmonella* death in soil samples in spring while natural soil bacteria competence was the main factor affecting in autumn. Soil was able to filtrate *Salmonella,* but preferential flux transported bacteria to 0.45m depth, surviving 3 weeks. Soil physical properties, irrigation system and water management will have an effect on the sanitary risk associated to reclaimed water irrigation. In this sense, SDI must be considered as the safest irrigation method.

Key word: Salmonella**,** survival, radiation, reclaimed water, soil

RESUME - *L'acceptabilité des eaux dépurées sur d'autres sources d'eau pour l'irrigation dépend de l'impact sur l'environnement et des risques sanitaires. En ce qui concerne ces derniers, les conditions spécifiques du site de réutilisation influence l'existence et persistance des pathogènes, l'information de la littérature scientifique à ce sujet étant contradictoire. La chloration lors du traitement de l'eau est très étendue mais il y a des évidences sur la survivance de Salmonella. L'objectif de ce travail est de contribuer à la connaissance de l'effet du climat et des sols sur la transport et la survivance de Salmonella dans des conditions de terrain en culture. On présente les résultats de trois expériences. Cinq pots semés de Medicago sativa furent irrigués avec un effluent secondaire inoculé de Salmonella. Les paramètres climatiques furent registrés avec une station climatique automatique. Les tiges et les feuilles, ainsi que les sols à trois profondeurs (surface, 0.15 et 0.45 m) furent échantillonnés. Comme résultat de la radiation, le nombre de bactéries décru pendant le jour, tandis que pendant la nuit une re-croissance se produit, spécialement dans l'expérience du printemps. Après trois semaines sur le terrain, on pouvait détecter la Salmonella dans les échantillons végétaux. La radiation semble être la cause de la mort de Salmonella dans les sols au printemps tandis que la compétence biotique pourrait être le facteur principal en automne. Le sol est capable de filtrer la Salmonella, mais à travers le flux préférentiel, elle peut atteindre le sous-sol (0.45 m de profondeur) où elle peut survivre au moins trois semaines.*

Mots Clés: *Salmonella, survivance, radiation, eau dépurée, sol*

INTRODUCTION

Municipal reclaimed water, as a non-conventional resource, can increase the sustainability of agricultural production mainly in arid and semiarid countries. Specific site conditions influence pathogens existence and persistence in agricultural lands (Guan and Holley, 2003). Water and soil characteristics, irrigation systems and water management (Sivapalasingam, 2003), crops, animal and human exposure affect so much the risk associated with water reuse that no quality standards are universally accepted (WHO, 1989 and 2000, Pescod, 1992, Westcot, 1997, Blumenthal *et al*., 2000, ANZECC, 2000, Carr *et al.,* 2004, USEPA, 2004) and sometimes contradictory information in the scientific literature is found based on the proposed criteria: risk assessment or sustainability criteria (Jensen *et al*., 2001). Finally, the climatic effect on pathogen persistence or attenuation must be also considered. Thus, acceptability of reclaimed water to replace other water resources for irrigation is highly dependent on whether the health risk and environmental impacts entailed (Pedersen *et al.*, 2005) are acceptable or not (Angelakis *et al*., 1999). There is a lack of regulatory standards in Europe regarding reclaimed or "regenerated" water reuse. In fact, nowadays in Spain there is not reuse regulations at all.

Total count and species of microorganisms founded in wastewater widely varies due to climate conditions, season, population sanitary habits and diseases incidence. The use of many bacteria indicators is extended, but only limited species of them are pathogens. *Salmonella* is one of the pathogenic bacteria most frequently associated with water caused illness (Fernández-Crehuet y Espigares, 1995; Fett and Cooke, 2003; Brooks *et al*., 2003; Estrada-Garcia *et al*., 2004). Although chlorination is widely extended, there are situations in which coli forms, *Salmonella* and other heterotrophic bacteria are able to survive (Al-Nakshabandi *et al*., 1997; Snelling *et al.*, 2006) especially in rural communities with low cost water treatment plants (Hernandez Moreno and Palacios, 2006). In this sense, Tejedor *et al.* (1998) detected the rare presence of *Salmonella* in chlorinated reclaimed water in agricultural field lands. That presence coincided with seldom high DBO_5 values of the secondary effluent measured in the reclaimed water treatment plant. *Salmonella* lives in gastrointestinal habitat. Thus, it is widely accepted that it has a limited survival period in environmental conditions. Despite of this, high nutrient contents of agricultural soils (especially when irrigated using reclaimed water) and favourable temperature and humidity conditions (frequent in irrigated lands from semiarid countries) have been mentioned as a suitable environment for pathogenic bacteria (Byappanahalli and Fujioka, 1998). In this sense, pathogenic bacteria have been previously cited as able to persist in water, soils and on crops (Batarsseh, *et al.* 1989a,b, Hassen *et al*., 1996), agreeing with our results showing that *Salmonella* is able to survive in reclaimed water ponds for periods longer than one month (Tejedor-Junco *et al.*, 2005) in subtropical climates.

The establishment of Macaronesian quality guidelines for reclaimed water (RW) reuse envisages: (i) Developing a single set of guidelines and criteria that are appropriate for Macaronesia and that are based on a consensus of Macaronesia expert and other role players in water quality, and (ii) Adapting national/international guidelines in the light of local research and experience (Palacios and Hernandez- Moreno, 2005). Thus, the aim of this experiment was to increase the scientific information on the climatic and soil effects on pathogen transport and survive under agricultural field conditions, in order to improve local knowledge and to establish the best reclaimed water management practices from health, environmental and economical point of view.

MATERIALS AND METHODS

Analysing the results of two previous assays that were carried out during autumn and spring (Palacios *et al*., 2001) we decided to conduct a third experiment. In this experiment we increased the frequency of sampling to demonstrate our hypothesis. Thus, the results from this third experiment carried out during the following autumn are presented here. Detailed material and methods are presented in mentioned paper. Five pots (0.8 m height and 0.9 m in diameter) were placed in the experimental field, filled with local soil 9 months before the first experiment and seeded with *Medicago sativa*. Two pots were irrigated using a secondary effluent in each experiment: one artificially inoculated and the second one without *Salmonella* (control). One control pot from the first experiment was reused for the third. Main soil characteristics were: clay soil (47% of expansive clay), 1.1% of organic matter, 1.8 dS/m of electrical conductivity, 38 and 83 mg/kg of nitrates and Olsen phosphorous and 4.36 me/100 g of potassium. An Automatic Weather Station recorded climatic

parameters. The reclaimed water were artificially inoculated with a known *Salmonella* biotype and serotype with $(124\pm8)\cdot10^3$ (first experiment) $(565\pm85)\cdot10^3$ (second) and $(412\pm73)\cdot10^3$ (third) cfu·mL^{-1}. Reclaimed water samples were collected in sterilized bottles before inoculation and were analysed for *Salmonella* presence. Soil and plant samples were also analysed before each experiment.

Composite plot soil samples were taken from soil surface, 0.15m and 0.45m depth, after different periods in each experiment: 2, 7 and 12 days after irrigation (first experiment); 22, 46, 166, 310 and 358 hours after irrigation (second experiment) and every two hours from sunrise to sundown during 3 days, 10 and 20 days after irrigation from the soil surface. For 0.15 and 0.45m soil a sample was taken 1, 2, 3, 10 and 20 days before irrigation (third experiment). Open plates (filled with sterilized soil) and ultra violet radiation isolated plates (filled with non sterilized soil) were sited on the two plots surface in the second and third experiments. Every plate was irrigated using the same quality of water than used to irrigate each plot using higher counts ($(5\pm1.5)\cdot10^6$.cfu·mL^{-1}) for the third experiment.

Composite plant samples were collected in each plot: 0, 1 and 2 days after irrigation during the first experiment, 0, 3.5, 11, 22, 46, 166 and 214 hours after irrigation (second one) and using the same frequency for sampling as described by the surface of the soil (third one).

Salmonella count was determined using Brillant Green and Rapport Agar plates incubated at 43 ºC. Plant and soil samples (25g) were diluted in 225mL peptone buffered water with increasing dilution values. Thus, the results are presented in cfu·mL^{-1}. Presence test were also made in all the samples for the second and third experiments. More detailed procedures, which were already described for second experiment, are presented in Palacios *et al.*, 2001.

RESULTS AND DISCUSSION

Salmonella ausence was found in all the water, soil and plant samples before the experiments. Likewise, *Salmonella* ausence and non detectable counts were found in every soil and plant samples irrigated with non inoculated water (control) during the experiments.

Results of *Salmonella* counts for the third experiment in plots irrigated with inoculated reclaimed water are shown in Fig. 1. Plant data from the first and the second experiment are also represented.

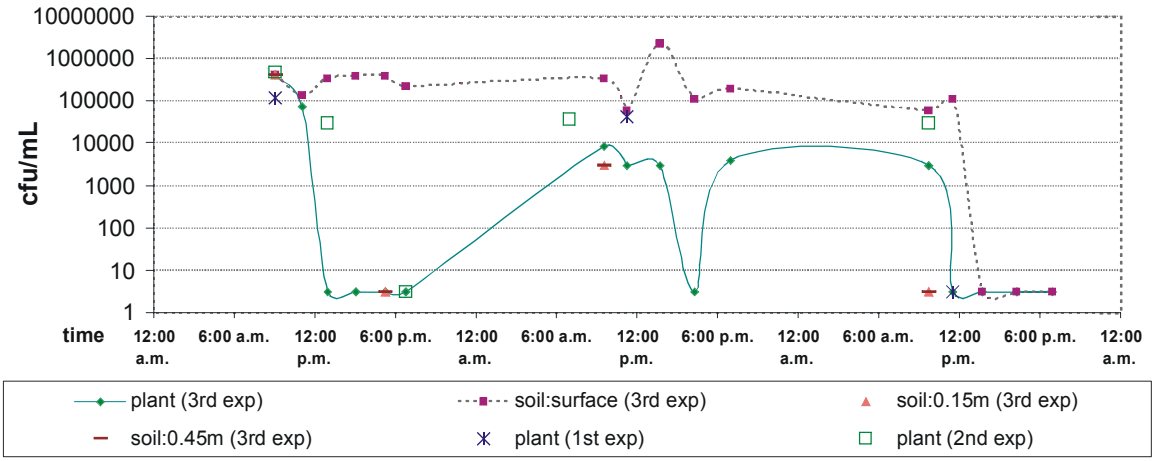

Fig. 1. Results of *Salmonella* counts obtained during the third experiment (November), in cfu·mL^{-1} (Artificially inoculated reclaimed water: $(565\pm85)\cdot10^3$ cfu·mL^{-1} (second experiment) and $(412\pm73)\cdot10^3$ cfu·mL^{-1} (third one) for soil and plant samples).

As result of the solar radiation effect, less bacteria counts were progressively obtained on plant (continuous line with lozenges in Fig. 1) over time during the day. In fact, since the first day following irrigation, some no detectable counts were obtained due to the presence of many environmental bacteria growing in the laboratory plates that difficult the detection of *Salmonella*, although presence

test detected it. These results were also obtained by the second experiment and are coincident with obtained by other authors (Turpin et al, 1993). In order to represent these detectable but not countable results, it was decided to assign them the value three.

During the night, Salmonella was able to grow although for 5 and 4 log(10) cfu·mL declines in the population organisms in plant samples from initially inoculated and from the first night population at field conditions respectively. These results were consistent for at least three days and for the three experiments demonstrating the solar radiation effect in bacterial death growing on the plant surfaces. Higher recovering was obtained during the spring nights of the 2nd experiment, probably due to the higher nocturnal temperatures (also affected by soil heat emission), from this season. *Salmonella* was detected for the 10 and 20 days before irrigation (3 and ($9 \cdot 10^3$) cfu·mL^{-1}, respectively). This result coincides with obtained by Ronconi *et al.*, (2002) who demonstrated the persistence of *E Coli* in lettuces. Similarly, Guo *et al.,* (2001) detected persistence of *Salmonella* in tomato plants and fruits over a longer period of time following inoculation at field conditions.

Comparing plant and soil results we noticed that bacteria survival was higher on the soil surface than on the plant samples. Although as occurred in plant samples an initial declination in bacterial populations was obtained from the sunrise to the noon, bacteria mortality was low and it was able to recover high populations even during the day. It is probably due to the solar radiation attenuation caused by plant and soil particles shading. Results obtained in subsoil samples (at 0.15 and 0.45m triangles and stripes in fig 1) are coincident with obtained in plant samples. Soil was able to effectively filtrate *Salmonella,* decreasing its population in 2 log (10) cfu·mL for the second day, but preferential flux may let the bacteria to reach subsoil at 0.45 depths. As obtained for the second experiment *Salmonella* seemed to need one day after irrigation to reach this depth. But, once in subsoil, it was able to survive at least 3 weeks in subtropical climates. This result is consistent which obtained by other authors (Ibenyassine *et al*., 2006).

Fig. 2 represents the results for the comparative study from the effect of *Salmonella* competence with soil bacteria to radiation. Filled lozenges and discontinuous line represent sterilized soil plates results with lozenges for the 2nd and 3rd experiments respectively, while ultra violet radiation blocked plates (filled with non sterilized soil) are represented by addition signs and continuous line with asterisks (2nd and 3rd experiments respectively). Radiation seems to be the main cause of *Salmonella* death in soil samples during the spring season (2nd experiment, with spring longer day duration) while natural soil bacteria competence could be the main factor affecting in autumn (3rd experiment), coinciding with Turpin *et al.* (1993). Hence, the season effect could explain contradictory results obtained by many authors. As latitude condition day duration too, the conclusions obtained with local survival studies have not to forget this factor. You *et al*. (2006) also founded higher *Salmonella* persistence when manure is applied to sterilized soil comparing to no sterilized one.

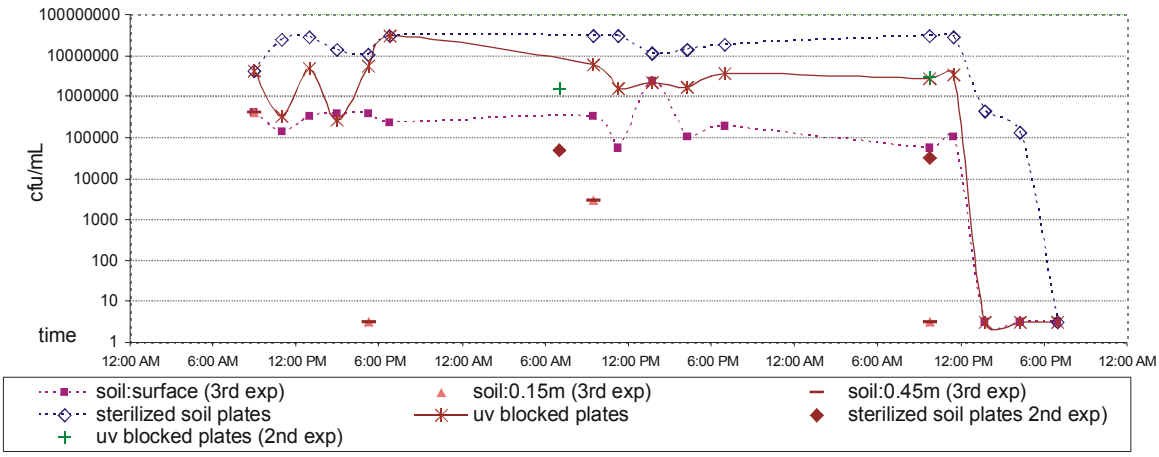

Fig. 2. Results of *Salmonella* counts obtained during the second (spring) and the third (autumn) experiments, in cfu·mL^{-1} (Artificially inoculated reclaimed water: (412 ± 73)·10^3 cfu·mL^{-1} for soil samples and (565 ± 85)·10^3) cfu·mL^{-1} for plates.

In the 3rd experiment, and when soil bacteria competence was eliminated, even an increase of 1 log (10) cfu·mL was measured during the first two days after irrigation. This result coincided with obtained by You et al. (2006) who founded a *Salmonella* concentrations increase by up to 400% in the first 1 to 3 days after inoculation following by a steady decline. Once again, the solar radiation attenuation caused by plant and soil particles shading, joined to competence elimination could explain this increment in *Salmonella* populations. Comparing the results obtained by surface soil and plates with subsurface soil (triangles and stripes, fig 2), and considering results obtained by Al-Nakshabandi *et al.* (1997) and Batarsseh, *et al.* (1989a,b) we concluded that soil bacteria "filtration" was the main factor to decrease pathogen risk associated with reclaimed water irrigation. Complex transport behavior other than advection-dispersion, simple retardation and first order removal has been observed in many biocolloid transport experiments in porous media (Cortis, *et al.,* 2006). Colloid transport in subsurface environments is critical to solving problems related to groundwater pollution by microbial pathogens (Saiers and Ryan, 2006). In this sense, subsurface drip irrigation systems (SDI) must be considered as the safer way to reuse reclaimed water. Contradictory results were obtained by Assadian *et al*., (2005) who concluded that preferential flow of irrigation water to the surface of clayey soil columns promoted virus movement to the soil surface when using SDI. In spite of this, many authors mentioned that associated with increasing irrigation frequency (comparing drip and furrow irrigation) less vertical cracking clay was observed (Hodgson *et al.,* 2005). This second experiment was carried out at field conditions, while Assadian *et al*. worked in soil columns.

CONCLUSION

High *Salmonella* levels artificially inoculated, higher than may be expected in any reclaimed water used for irrigation, were used in these studies. Analysing the results obtained by this 3rd experiment we are now able to demonstrate our previous hypothesis: *Salmonella* was able to survive at least 3 weeks on plant, surface and subsurface soil samples at field conditions in subtropical climates. Soil was able to effectively filtrate *Salmonella,* but preferential flux may let the bacteria reach subsoil at 0.45m depth. Due to this fact, soil physical properties, irrigation system and water management will have an effect on the sanitary risk associated to reclaimed water irrigation. In this sense, SDI must be considered as the safest irrigation method. The solar radiation declined pathogen's populations from initially inoculated on plant and soil surface during the day. In spite of this, during the night *Salmonella* was able to re-grow, basically depending on the nocturnal temperatures, also affected by the soil heat emission. Radiation was the main cause of *Salmonella* death in soil samples in spring while natural soil bacteria competence was the main factor affecting in autumn, when the solar radiation attenuation caused by plant and soil particles shading seemed to be a critical factor.

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