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Standardization of a quantification method for *Salmonella* spp. *and Shigella* spp. in specific liquid media

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SUMMARY

Introduction: Chlorination is the most widely used disinfection process for drinking water production. The formation of chlorination carcinogenic by-products and chlorine intoxication by direct manipulation in small communities has motivated the study of alternative disinfection processes. In this sense, processes of advanced oxidation (PAOs) have yielded promising results. *Escherichia coli (E. coli)* is customarily used as faecal bacterial indicator to determine the efficiency of disinfection processes. However, it has been shown that *E. coli* is less resistant to disinfection than other enteric bacteria such as *Shigella* spp. and *Salmonella* spp. Additionally, the viable non-culturable (VNC) state yields bacteria which are not detectable on many culture media.

Objective: The main objective is to standardize a method for counting *Salmonella* spp. and *Shigella* spp. in specific liquid media to reliably quantify the bacteriological potential risk related to disinfection processes based on PAO.

Methods: The study followed a randomized bi-factorial experimental design and the Duncan multiple comparison test. This design allowed the selection of specific liquid media to fittingly standardize the counting of *Salmonella* spp. and *Shigella* spp.

Results: We found that the best broth for counting *Salmonella typhimurium* strain at different concentrations in pure and mixed cultures was the *Rappaport broth* RP, the EE broth also allowed growing the two bacterial species tested in this research. Nonetheless, the latter results suggest the use of additional tests for this particular broth.

Discussion: There was a variation in the counting results when pure cultures were used compared to those obtained from mixtures of microorganisms. It was also noted that *Salmonella typhimurium* and *Shigella sonnei*, were recovered from minimal concentrations in both RP and EE broths, respectively. To some extent, this suggests an additional confirmative method when using the EE® broth.

Conclusion: MPN is a rapid and inexpensive method; easy to apply in water and other contaminated environments where counting of *Shigella* spp. and *Salmonella* spp. is needed to estimate potential bacteriological risks. The broths selected were able to recover the two bacterial species from densities as low as 10 cells per 100 ml.

Keywords: *Bacterial quantification; Shigella spp.; Salmonella spp.; Most probable number (MPN); Advanced oxidation process; Faecal pollution indicators.*

Estandarización de un método de recuento para Salmonella spp. y Shigella spp. en medios de cultivo líquidos especializados

RESUMEN

Introducción: La cloración es el método más usado para desinfectar aguas de consumo. La formación de subproductos cancerígenos y las intoxicaciones por manipulación directa en pequeñas comunidades, han motivado el estudio de procesos alternativos. Los procesos de oxidación avanzada (PAOS), han arrojado resultados prometedores, utilizando el indicador bacteriano *Escherichia coli* (*E. coli*), con el método recuento en placa. Sin embargo, también se ha demostrado que *E. coli* es menos resistente a la desinfección que otras bacterias entéricas como *Shigella* y *Salmonella* y que estos procesos generan bacterias viables que no se cultivan durante el proceso, y no se descubren en medios sólidos.

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Objetivo: Estandarizar un método de recuento de *Salmonella* sp. y *Shigella* sp., en medios de cultivo líquidos especializados, que permita valorar de forma confiable el riesgo bacteriológico en procesos de desinfección PAOS.

Métodos: En el presente trabajo se ensayaron y seleccionaron medios líquidos especializados, con los que se estandarizó el recuento de *Salmonella* sp. y *Shigella* sp., mediante un diseño experimental aleatorizado bifactorial y la prueba de comparaciones múltiples de Duncan.

Resultados: Se encontró que el mejor caldo para recuperar a *S. typhimurium* a diferentes concentraciones, en cultivos puros y mezclas, fue el caldo *Rappaport* de Merck (RP). El caldo de enriquecimiento para entero bacterias de Oxoid (EE), permitió un buen crecimiento de las dos especies objeto de esta investigación. Lo cual sugiere el empleo de pruebas adicionales cuando se use caldo EE para NMP.

Discusión: Se observó una variación en el recuento cuando se usaron cultivos puros, comparado con la obtenida a partir de mezclas de microorganismos. Sin embargo, *S. typhimurium*. y *Shigella* sonnei logran ser recuperadas de concentraciones mínimas en los caldos RP, respectivamente.

Conclusión: Se pudo estandarizar un método de fácil aplicación a aguas y otros ambientes contaminados para recuento de *Salmonella* sp y *Shigella* sp. Los medios líquidos seleccionados fueron capaces de recuperar concentraciones de menos de 10 bacterias.

Palabras clave: *Cuantificación de Shigella spp.; Salmonella spp.; Número más probable (MPN); Proceso de oxidación avanzada; Indicadores de contaminación.*

As a result of human activities and of the accelerated demographic growth, progressive deterioration of the quality of natural sources of water has been generated; this propitiate the development of an important diversity of pathogenic organisms, including viruses, bacteria, protozoa, fungi, and parasitic worms that represent risks, especially for rural communities where the coverage and quality of potabilization services is defficient[1]. Disinfection processes are not always present in waters consumed by these communities and in instances where they do exist; there are difficulties in the access to the disinfecting agent, in its storage, manipulation, and in the lack of technical capacity to carry out the process[2]. Disinfection with chlorine, also presents the risk associated to the generation of toxic compounds or disinfection by-products (DBPs) as a result of its reaction with the organic matter present[3], consequently, interest has been generated to reduce the chronic effects of these types of compounds without compromising the microbiological safety of the water[4] and different alternatives to disinfecting with chlorine have been explored, as are: solar radiation, electrochemical disinfection, ozonization, and advanced oxidation processes (AOPs)[5]. AOPs are processes involving the generation of highly oxidizing species often called reactive oxygen species (ROS), as for example hydroxyl radicals (•OH) with higher oxidation potential (E=2.8 V *vs*. NHE). This radical can be generated through photochemical means (UV and solar light) or through other forms of energy, and it is highly effective for the oxidation of organic matter[6]. Organic compounds are mainly oxidized through abstraction of hydrogen or by electrophilic addition to double bonds generating free organic radicals (OR) that react with oxygen molecules forming a peroxy-radical, initiating a series of oxidative degradation reactions that can lead to the thorough mineralization of the contaminants[7]. For the specific case of microorganisms, it has been reported that these radicals attack the double bilipid layer that makes up the outer cell wall generating reactions of lipid peroxidation that are lethal for microorganisms[8]. Photo-catalysis bears economic and ease-of-handling advantages given that it utilizes solar energy for the elimination of chemical compounds and inactivation of microorganisms[5].Because *E. coli* and the Coliforms group are the models of greater application for the estimation of the microbiological quality of the water under current regulations, numerous disinfection results with photo-catalysis have been published basing the conclusions on studies with these bacteria, using the quantification method on solid media. It has been shown that these processes can generate nonculturable viable bacteria, which can reactivate hours after treatment[9]. Furthermore, it is evidently important to prove the effectiveness of the process in other pathogens that could be more resistant to disinfection by using PAOs[10]. *S. typhimurium* and *Shigella sonnei* have important implications as causal agents of acute diarrheic illness (ADI). A study developed in 18 departments of Colombia, between January 2000 and December 2001, found an important incidence of ADI related to these bacteria[11]; additionally, in the department of Valle del Cauca, contamination of natural water sources has been observed with *Salmonella* and *Shigella*, and patients diagnosed with ADI have had these bacteria. There are no methods developed to quantify the genre of *Salmonella* spp. and *Shigella*

spp.; the methods available are applications to determine the presence or absence of the pathogens in a sample volume or quantification on plate in media like SS. Notwithstanding the great variety of products commercially available, the percentages of recovery may vary for the different formulations. Bearing this in mind, we consider it important to quantify these bacteria to evaluate the effectiveness of disinfection in the photo-catalysis process. The quantification with MPN has the advantage of favoring the growth of stressed cells and contributing a better reference of the process of disinfection with photo-catalysis [12], this technique permits establishing populations with ranges <3 CFU mL^{-1}, using relatively large sample volumes. The objective of the current work consisted in standardizing a low-cost methodology, based on the quantification technique with MPN to detect populations of *Shigella* spp. and *Salmonella* spp. The table of probabilities was obtained of MPN for *Salmonella* in Rappaport broth (Merck) and EE broth (Oxoid) for *Shigella*. A variation was observed in the quantification when pure cultures were used compared to those obtained from mixes of bacteria from different species. The liquid media selected were able to recover concentrations of less than 10 bacteria.

METHODS

Bacterial strains. For the study, ATCC reference strains were used; *Salmonella typhimurium* (*S. typhimurium*) 15,490 serovariety, *Shigella sonnei* (*S. sonnei*) 25,931, *Escherichia coli (E. coli)* 23,716, and *Staphylococcus aureus (S. aureus)* 6,538. For productivity and selectivity, *Klebsiella pneumoniae (K. pneumoniae)* was also used, isolated from clinical specimens at Hospital Universitario del Valle, confirmed through biochemical tests in enterotubes (BBL II ref. 273,176), plastic tubes with compartments, formed by 12 different culture mediums, permitting the determination of 15 biochemical reactions.

Productivity and selectivity. In order to select the best culture broth to standardize the most-probable-number technique (MPN), to quantify *Salmonella* spp. and *Shigella* spp., four culture broths were used for experimentation: *Rappaport* broth from Merck (RP), enrichment broth for entero bacteria (EE) -Oxoid, selenite-cystine (SC) broth from Merck, and tetrathionate (TT) broth from Difco, recommended in standard methods for these bacteria and used in Colombia.

Productivity tests. 100 µl of bacterial suspension from each bacterial strain, was transferred to a tube with 10 ml of nutrient broth (Oxoid), it was incubated for 18 hours at 35ºC. With cultures from the *Shigella* spp. strain or *S. typhimurium* in stationary phase, (10^8 CFU ml^{-1}, adjusted with the MacFarland Standard at 0.5) scale, consecutive decimal dilutions (10^{-1} a 10^{-10}) were prepared, with two repetitions, in peptonated water at 0.1%. We seeded 1 ml from each dilution in tubes with 10 ml of the RP, EE, SC, and TT media; the tubes were incubated at 35°C. *E. coli* and *S. aureus* were used as control strains. The productivity of a selective culture broth should permit the development of a pure culture of the strain desired with an inoculate of 20 cells or less[13]; thus, the number of CFU ml^{-1} was estimated from the last two positive dilutions taking 0.1 ml to growth on plates of nutrient agar, Hektoen medium from Merck was used for *S. sonnei*, xylose-lysine deoxycholate (XLD) from Merck for *S. typhimurium*, incubated at 35°C for 24 hours.

Selectivity test. What is sought with this test is to determine the capacity of a culture medium to inhibit accompanying biota[14]. *E. coli* ATCC 23,716, *S. aureus* ATCC 6,538, and *K. pneumoniae* strains were used in the study. 1 ml of culture in stationary state of each of the microorganisms was transferred onto a test tube. From the mixture, a 10^{-2} dilution was prepared in peptonated water at 0.1%. For the test strains (*S. sonnei* and *S. typhimurium)*, 10 consecutive dilutions (10^{-1} - 10^{-10}) were done. In tubes with 10 ml of each of the four culture mediums, RP, EE, SC, and TT, 0.2 ml of the mixture and 1 ml of each dilution of the test strains was seeded; it was incubated at 35±1°C between 18-24 hours, the culture in SC was incubated at 44±1°C. From 0.1 ml of the 3 last positive dilutions and a negative dilution, the CFU ml^{-1} number was estimated in agar plates with Oxoid Nutrient, Hektoen from Merck, Levine eosin methylene blue agar EMB medium, and xylose-lysine deoxycholate medium from Merck (XLD). The Petri dishes were incubated at 35±1°C for 24 hours. Quantification and biochemical tests were conducted on the resulting colonies in BBL enterotubes.

Interpretation parameters. For the qualitative and quantitative assessment of productivity and selectivity, we kept in mind the recommended parameters for international standards[15] that were also employed in

Table 1
Factors and levels of the randomized bi-factorial structure experimental design for the standardization of the MPN

Factors	Levels
First factor: Microorganisms	Shigella sonnei in EE broth (A), *Staphylococcus aureus*. in EE broth (B), Mix. in EE broth (C), *Escherichia coli* in EE broth (D), *Salmonella* ser *typhimurium* in Rappaport broth (E), *Staphylococcus aureus.* in Rappaport broth (F), Mix. in Rappaport broth (G), *Escherichia coli* in Rappaport broth (H).
Second factor: Bacterial concentration	10^1, 10^2, 10^3, and 10^4

Colombia. We compared turbidity and the quantification of CFU ml-1 $\geq$10.

Standardization of the most probable number. The MPN technique was standardized for *S. typhimurium* and *S. sonnei*, taking as a model the protocols reported by the official Mexican norm, Nom-004-Sermanat of 2002[16], and by the MPN for *E. coli* according to standard methods of 2005, using liquid media RP and EE.

Experimental analysis. A randomized bi-factorial experimental design was used. The study considered 2 factors: A) Microorganisms that will comprise levels *S. sonnei* and *S. typhimurium* and the controls of the experiment; *S. aureus* (negative control), *E. coli* and a bacterial mixture composed of *E. coli, S. sonnei,* and *S. typhimurium.* B) The bacterial concentration, which comprises levels 10^1, 10^2, 10^3, and 10^4 for a total of 32 treatments (Table 1). The experimental units considered in this study are the test tubes with the broths chosen based on the results in the productivity and selectivity phase. For each test tube containing the different broths (RP and EE) different treatments are randomly assigned; *S. typhimurium* and *S. sonnei* and controls with their respective concentrations. With the results from the first 16 experiments of the MPN, representativeness of the sample size was analyzed. Finally, the probability tables were found according to Briones *et al*., based themselves on the data by deMan[17]. The SPSS statistical package, version 10 of 2006, was used for data processing.

RESULTS AND DISCUSSION

Selection of culture media to standardize the MPN technique for S. typhimurium and S. sonnei. The MPN technique has been used over several years to quantify *E. coli* and other microorganisms. One of the most critical aspects of this method is the selection of reliable culture media, which permit the development of the desired microorganism, but inhibit the accompanying micro flora. For desired microorganisms the quantification should be around 10^6 CFU ml^{-1} to 10^8 CFU ml^{-1}. For undesired microorganisms, the count should not exceed 10^4 CFU ml^{-1} [13]. Symbols were given (-) for negative turbidity and negative growth on plate (+), for positive turbidity and positive growth. Commercially, there is no availability of dehydrated culture media that are selective for *Shigella* spp.; for this reason, measurements for these bacteria were complemented with biochemical tests.

The results are shown in Tables 2 and 3, noting that although the productivity of all the broths for the recovery of suspensions of *S. typhimurium* in pure culture is high, when in the presence of other microorganisms recovery may vary, the highest growth values were obtained in RP -this broth was productive and selective for *S. typhimurium* up to concentration 10^9- no growth of *S. aureus* was observed. Productivity of *S. sonnei* and *E. coli* were low in this culture medium.

In the SC broth, *S. typhimurium* productivity was high, growing in concentrations of 10^9 bacteria per ml^{-1}, but selectivity was low and high concentrations of

Table 2
Results of productivity tests for sample bacteria (*S. typhimurium*, *S. sonnei*) and control bacteria

	RP	SC	EE	TT	RP	SC	EE	TT
Liquid media dilutions	*S. typhimurium*				*S. sonnei*			
10^1	+	+	+	+	+	+	+	+
10^2	+	+	+	+	-	-	+	-
10^3	+	+	+	+	-	-	+	-
10^4	+	+	+	+	-	-	+	-
10^5	+	+	+	+	-	-	+	-
10^6	+	+	+	+	-	-	+	-
10^7	+	+	+	+	-	-	+	-
10^8	+	+	+	+	-	-	+	-
10^9	+	+	+	-	-	-	-	-
10^{10}	-	-	-	-	-	-	-	-
	E. coli				*S. aureus*			
10^1	+	+	+	+	-	-	-	-
10^2	+	+	+	-	-	-	-	-
10^3	+	-	+	-	-	-	-	-
10^4	+	-	+	-	-	-	-	-
10^5	+	-	+	-	-	-	-	-
10^6	+	-	+	-	-	-	-	-
10^7	-	-	+	-	-	-	-	-
10^8	-	-	+	-	-	-	-	-
10^9	-	-	-	-	-	-	-	-
10^{10}	-	-	-	-	-	-	-	-

Rappaport (RP). Enrichment broth for entero bacteria (EE). Selenite-cystine (SC). Broth and tetrathionate broth (TT).
Sign (+) Turbidity with confirmed quantification >10 CFU; Sign (-) No turbidity, or growth in the solid medium

K. pneumoniae were detected in dilution concentrations of 10^{10} bacteria per ml^{-1}. This broth permitted growth of *S. sonnei* in concentration of 10^1 and *E. coli* up to 10^2 bacteria per ml^{-1}.

In the medium, EE showed high productivity for *S. sonnei* which grew up to concentration 10^8, *S. typhimurium* grew to concentration 10^9 bacteria per ml^{-1}, and *E. coli* grew to concentration 10^8 bacteria per ml^{-1}. Selectivity results for *Shigella* in this medium were comparatively better than in the other broths. *S. sonnei* and *S. typhimurium* grew to concentration 10^8 bacteria per ml^{-1}; this broth permits recovery of entero bacteria in general, it was shown that this is a selective medium for entero bacteria and it is not differential, which is explained by the high percentages of simultaneous *E. coli* and *S. typhimurium* recovery. The negative control (*S. aureus)* did not grow in the media tested.

The TT broth revealed high productivity for *S. typhimurium* (10^8 bacteria per ml^{-1}); however, we also observed growth in high concentrations of *K. pneumoniae* 10^{10} bacteria per ml^{-1}.

Finally, it was found that the best broth for *S. typhimurium* recovery, considering tests of pure cultures and tests in mixtures with accompanying microflora, was RP. The EE broth permitted good growth for *S. sonnei* in pure cultures and in mixtures with accompanying microflora. Nevertheless, *S. typhimurium* was also recovered from this medium. This suggests the usefulness of this medium in simultaneous quantification of these two groups, but it should be complemented with biochemical tests. Since the specificity of RP, such could be used in a second confirmative step in plates of

Table 3
Selectivity tests for sample bacteria *S. typhimurium* and *S. sonnei*

	RP	SC	EE	TT	RP	SC	EE	TT
Liquid media dilutions	*S. typhimurium*				*S. sonnei*			
10^1	+	+	+	+	-	-	+	-
10^2	+	+	+	+	-	-	+	-
10^3	+	+	+	+	-	-	+	-
10^4	+	+	+	+	-	-	+	-
10^5	+	+	+	+	-	-	+	-
10^6	+	+	+	+	-	-	+	-
10^7	+	+	+	+	-	-	+	-
10^8	+	+	+	+	-	-	+	-
10^9	+	-	-	-	-	-	-	-
10^{10}	-	-	-	-	-	-	-	-

Rappaport (RP). Enrichement for enterobacteria (EE). Selenite-cystine (SC) broth and tetrathionate broth (TT). Sign (+) Turbidity with quantification of desired microorganisms in a range between 10^6-10^8 CFU/ml, and a quantification of undesired microorganisms, without exceeding 10^4 CFU/ml. Sign (-) Turbidity, but quantifications of desired and undesired microorganisms were within the range or did not grow.

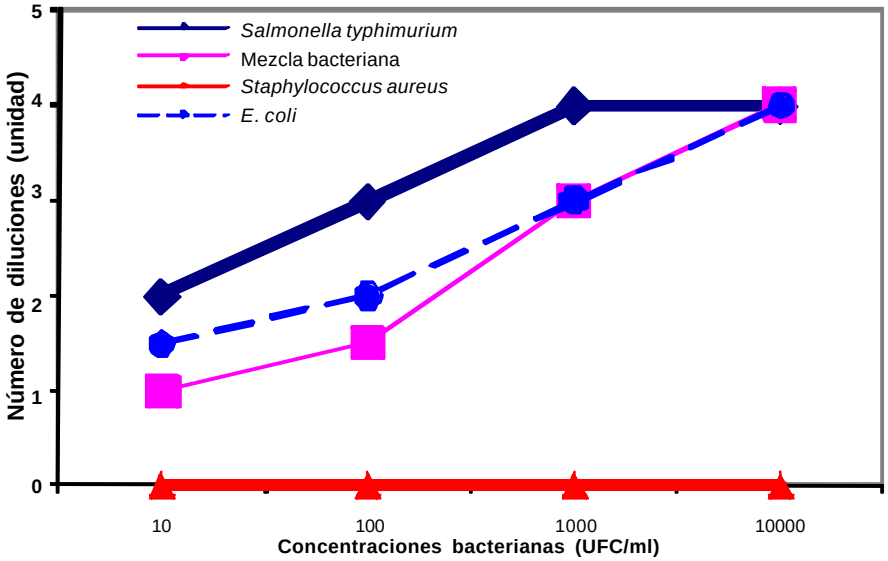
solid medium to separate counts.

Standardization of the MPN. In order to assess the experimental error and define the number of repetitions, tests were conducted in a series of 30 tubes for both microorganisms tested. No significant variability was observed among the repetitions for each treatment and within such, when only one repetition was used in each test. For the model established, it was found that there was good experimental control with 98.6% reliability for *S. typhimurium* and 99.3% for *S. sonnei*.

Analysis of the most probable number for S. typhimurium. Table 4 shows the analysis of the most probable number for *S. typhimurium* and its respective controls (bacterial mix, *E. coli*, and *Staphylococcus aureus)*. For the interaction hypothesis between concentration and microorganisms, statistic (F=12,667) found that the interaction between both factors considered is significant on the number of dilutions with p=0.0001, (r=0.986, p<0.0001), which is lower than for the significance (p=0.05), for S. *typhimurium* and for *S. sonnei,* statistic (F=22,333) found that its effect is significant over the number of dilutions with p=0.0001, (r=0.993, p<0.0001), which is lower than for the significance (p=0.05). Leading us to conclude that there is an interaction between these two dilution and microorganism factors (dependence); hence, it is not possible to analyze them independently. Bearing this in mind, to carry out the comparisons of the principal effects the microorganism level was set and the bacterial concentrations were compared by means of the Duncan multiple comparison test (Post Anova) for the number of dilutions, in the designs established of *S. typhimurium* and *S. sonnei*, respectively.

When performing the Duncan multiple-comparison test for *S. typhimurium*, in concentrations of 10^1 bacteria per ml^{-1}, the result is the same as when simultaneously culturing *S. typhimurium, S. sonnei,* and *S. aureus* or *E. coli* and *S. typhimurium.* However, differences were found from the dilution of 10^2 bacteria per ml^{-1}, yielding a significance level of (p=0.05). The inhibition of *S. typhimurium* and the rest of bacteria (*E. coli* and *S. sonnei*) in the bacterial mix control may be due to competitive effects among populations for the substrate. We observed that the effect of *S. aureus* is statistically different to the rest of the microorganisms, meaning that the bacteria did not grow in RP, as was expected, because said broth is selective for Gram negative bacteria like *Salmonella* spp. *S. aureus* was taken as negative control in the standardization of the MPN.

Upon analyzing concentration 10^2 with level of



Graph 1. Interaction between bacterial concentration and microorganisms for the number of dilutions for *S. typhimurium*

significance (p=0.05), it was found that the effect of *S. aureus* is different to that of *E. coli*, *S. typhimurium*, and the bacterial mix control. Since it was also established that the bacterial mix control has an equal effect as *E. coli* over the number of logarithmic units from which growth is obtained, *i.e.*, when *S. typhimurium* is in the microorganism mixture it tends to behave like *E. coli* when it is in pure culture. It was found that this bacterium grows to 10^3 in pure culture, better than when it is part of the bacterial mixture. It is important to highlight that in spite of the differences, *S. typhimurium* grows well in pure culture or in the presence of the accompanying microflora. The RP broth presents high selectivity to recover *S. typhimurium*. Results obtained by Lemarchand[18], in a study conducted in France where a correlation of fecal indicators, *Salmonella* spp. and *Cryptosporidium* spp., was carried out in contaminated coastal waters, found that it is feasible to quantify *Salmonella* spp. with the MPN method using the RP broth, which confirms the results obtained in this study.

For bacterial concentrations at 10^3 and 10^4, it was not necessary to carry out the multiple comparison test; since no difference was observed in the effect among *E. coli*, *S. typhimurium*, bacterial mix, and *S. aureus*.

In the concentration at 10^3, the microorganism mix, as well as *E. coli* grows three logarithmic units; different to *S. typhimurium*, which grows four logarithmic units. This result is very similar to what was obtained in the concentrations at 10^1 and 10^2. *S. aureus* did not grow because it is Gram positive.

In the concentration at 10^4, *S. typhimurium* grew four logarithmic units, both in pure culture as when it was part of the mix, *E. coli* also grew four logarithmic units. That is, there is a direct relationship between the dilution used and the growth obtained (Graph 1, Table 4). As in the previous concentrations *S. aureus* did not grow in equal manner as in the previous concentrations.

We can conclude that the RP broth permits good recovery of *S. typhimurium*; coinciding with Moreno *et al.*, who used the RP broth as enrichment broth for *Salmonella* spp. from samples taken at a secondary treatment plant of residual waters. This pre-enrichment increased the number of viable cells detectable with *in situ* hybridization[19].

Analysis of the most probable number for S. sonnei. The analysis for the most probable number for *S. sonnei* is presented in Tables 6 and 7. As for *S. typhimurium*, its respective controls are presented: *E. coli*, bacterial mix, and *Staphylococcus aureus*.

Analysis of the model of the S. sonnei design. For all bacterial concentrations from 10^1 - 10^4, it was not necessary to carry out the multiple comparison test,

Table 4
Analysis of the most probable number for *S. typhimurium*

Index of most probable number (IMPN) with 95% reliability limit for 5 tubes and three dilutions in series

Tubes (+)					MPN/ML	Reliability limits 95%		Bacteria	Concentration	Estimated population
5 of 10^{-1}	5 of 10^{-2}	5 of 10^{-3}	5 of 10^{-4}	5 of 10^{-5} 5 of 10^{-6}		INF	SUP			
5	5	0	0	0	239.789	79.725	722.637	*S. typhimurium*	10^1	2.40E+03
5	0	0	0	0	23.116	7.814	68.461	Mix	10^1	2.31E+02
5	5	0	0	0	239.789	79.725	722.637	*E. coli*	10^1	2.40E+03
0	0	0	0	0	<3			*S. aureus*	10^1	
5	5	5	0	0	2397.712	797.554	7228.55	*S. typhimurium*	10^2	2.40E+05
5	5	0	0	0	239.789	79.725	722.637	*Mix*	10^2	2.40E+03
5	5	0	0	0	239.789	79.725	722.637	*E. coli*	10^2	2.40E+03
0	0	0	0	0	<3			*S. aureus*	10^2	
5	5	5	5	0	23908.211	7966.959	72007.798	*S. typhimurium*	10^3	2.40E+07
5	5	5	0	0	2397.712	797.554	7228.55	*Mix*	10^3	2.40E+05
5	5	5	0	0	2397.712	797.554	7228.55	*E. coli*	10^3	2.40E+05
0	0	0	0	0	<3			*S. aureus*	10^3	
5	5	5	5	0	23998.255	7982.482	72410.23	*S. typhimurium*	10^4	2.40E+07
5	5	5	5	0	23908.211	7966.959	72007.798	*Mix*	10^4	2.40E+07
5	5	5	5	0	23908.211	7966.959	72007.798	*E. coli*	10^4	2.40E+07
0	0	0	0	0	<3	-	-	*S. aureus*	10^4	

because there is no difference in the effect of *E. coli*, *S. sonnei*, mix of species, and *S. aureus. S. sonnei*, in EE broth, like *S. typhimurium* in RP, grows better in pure culture than when it is accompanied by other species. The mixture of microorganisms and *E. coli* show the same behavior, requiring the same dilutions to grow in EE. The *S. aureus* control did not grow (Figure 2). Our results confirm those of Warren[20], who compared conventional culture methods and the polymerase reaction (PCR) to detect *Shigella* spp. in tomato surfaces, finding that the EE broth recovers *Shigella* spp. very well from high concentrations at 10^5 to vary low ones at 10^1.

There is no variation between the mixed culture and *E. coli*; the quantification is proportional to the dilution of origin (Graph 2, Table 5).

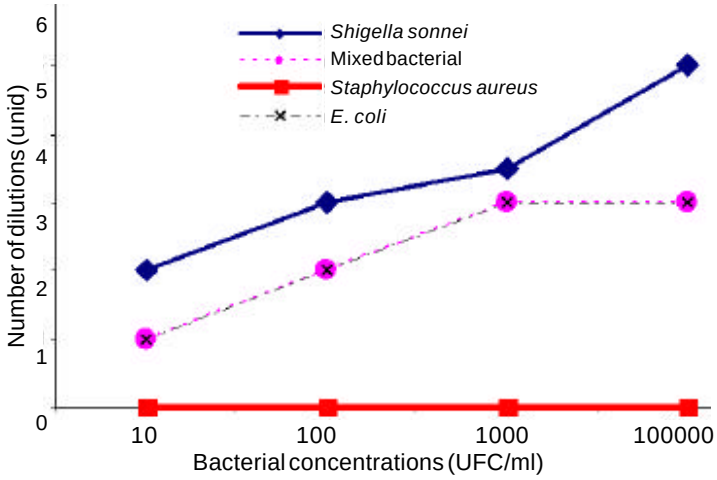
S. sonnei grows better in pure culture; this result was expected because bacteria in mixed culture a) can compete for the nutrients, and/or b) their metabolic products inhibit growth. The concentrations at 10^1 and 10^2 of *S. typhimurium* are detected in the RP broth, but when such bacterium is in a mixed culture, (*S. sonnei*, *S. typhimurium*, and *E. coli)* it is inhibited. *E. coli* grows well in the RP broth, even if inoculated at low concentrations of 10^1 and 10^2. *S. sonnei* tends to grow in greater concentrations than *E. coli* in pure culture.

The EE broth permits good

Table 5
Relation between CFU and detection limit in Rappaport (Merck) liquid medium

	CFU/ml			
	10^1	10^2	10^3	10^4
Salmonella typhimurium serovariety	0.01	0.001	0.0001	0.0001
E. coli	0.5	0.01	0.001	0.0001
S. aureus	ND	ND	ND	ND
Bacterial Control Mix	0.1	0.5	0.001	0.0001

ND: Not detectable



Graph 2. Interaction between bacterial concentration and microorganisms for the number of dilutions for *S. sonnei*

growth of the *S. sonnei* bacterium, both in pure culture and mixed culture. Bacterial identification was verified via manual biochemical tests (BBL). *S. aureus* did not grow in this medium.

When conducting the MPN in EE broth with concentration at 10^1 of *S. sonnei* we found that said bacterium grows from two dilutions, in the standardization of the MPN when the bacterium is part of the mixture (*S. sonnei*, *E. coli*, and *S. typhimurium*) it only grows from the first dilution, similar to that found with *E. coli*. When *S. sonnei* was inoculated from the concentration at 10^2 in EE broth, it grew by 3 logarithmic units; when the bacterium is in a mixed culture, it grows by two logarithmic units, as does *E. coli*. When conducting MPN with the concentration at 10^3 of *S. sonnei*, it was found that it grew by 3.5 and 3 logarithmic units in pure culture and in mixed culture, respectively, as does *E. coli*. When MPN is conducted with a concentration at 10^4, it was found that *S. sonnei* grows by 5 logarithmic units; while in mixed culture it grows by 3 logarithmic units.

CONCLUSIONS

The best results for recovery of different cellular densities of *S. typhimurium* were obtained in the Rappaport broth from Merck (RP) and for *S. sonnei* in enriched broth for entero bacteria from EE (Oxoid). For standardization tests, the Duncan multiple tests permitted establishing the differences in the recovery of the microorganisms tested for the different cellular densities; greater recovery was observed for all cellular densities in pure cultures and significant differences were established with their recovery in the presence of other bacteria species. Given that in nature and in real samples there is also the coexistence of a diversity of bacteria, we should expect relationships similar to competition and/or inhibition. However, even if the bacteria are accompanied, they manage to grow in minimal concentrations in the respective broths.

The *S. sonnei* and *S. typhimurium* bacteria could be detected and quantified in their respective EE and RP broths when residual and domestic waters have been treated for disinfection with UV, processes of advanced oxidation, Ozonization, and SODIS, which are methods that can generate viable nonculturable bacteria due to oxidative stress. Notwithstanding, given that the EE broth (Oxoid) permits good recovery of *S. sonnei* -both in pure culture as in bacterial mixtures, but it is not

Table 6
Analysis of the most probable number for *S. sonnei*

Index of most probable number (IMPN) with 95% reliability limit for 5 tubes and three dilutions in series										
Tubes (+)						MPN/ML	Reliability limits 95%		Bacteria	Concentration	Estimated population
5 of 10^{-1}	5 of 10^{-2}	5 of 10^{-3}	5 of 10^{-4}	5 of 10^{-5}	5 of 10^{-6}		INF	SUP			
5	5	0	0	0	0	239.789	79.725	722.637	*S. sonnei*	10^1	2.40E+03
5	0	0	0	0	0	23.116	7.814	68.461	*Mix*	10^1	2.31E+02
5	0	0	0	0	0	23.116	7.814	68.461	*E. coli*	10^1	2.31E+02
0	0	0	0	0	0	<3			*S. aureus*	10^1	
5	5	5	0	0	0	2397.712	797.554	7228.55	*S. sonnei*	10^2	2.40E+05
5	5	0	0	0	0	239.79	79.725	722.638	*Mix*	10^2	2.40E+03
5	5	0	0	0	0	239.79	79.725	722.638	*E. coli*	10^2	2.40E+03
0	0	0	0	0	0	<3			*S. aureus*	10^2	
5	5	5	0	0	0	2397.712	797.554	7228,55	*S. sonnei*	10^3	2.40E+05
5	5	5	0	0	0	2397.712	797.554	7228.55	*Mix*	10^3	2.40E+05
5	5	5	0	0	0	2397.712	797.554	7228.55	*E. coli*	10^3	2.40E+05
0	0	0	0	0	0	<3			*S. aureus*	10^3	
5	5	5	5	0	0	226103.362	77297.477	664321.525	*S. sonnei*	10^4	2.26E+09
5	5	5	0	0	0	2397.865	797.58	7229.243	*Mix*	10^4	2.40E+05
5	5	5	0	0	0	2397.865	797.58	7229.243	*E. coli*	10^4	2.40E+05
0	0	0	0	0	0	<3			*S. aureus*	10^4	

selective for *S. typhimurium.* This medium can be used as a differential always accompanied by confirmation tests in solid media.

The MPN is a method of easy application and extensive use, which can be used based on the results to economically and reliably quantify *S. typhimurium* and *S. sonnei* in disinfection processes.

Conflict of interest. None of the authors has conflicts of interest related to this study.

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Table 7
Relation between CFU and limit of detection in the EB liquid medium

	CFU/ml			
	10^1	10^2	10^3	10^4
S. sonnei	0.01	0.001	0.0001	0.00001
E. coli	0.1	0.01	0.001	0.001
S. aureus	ND	ND	ND	ND
Bacterial Control Mix	0.1	0.01	0.001	0.001

ND: Not detectable

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