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Identification of Bacteria in Water for Pharmaceutical Use

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ABSTRACT. Different systems for the obtention of water used in Biopharmaceutical Industry were characterized from the bacteriological point of view. Determination of aerobic mesophilic microorganisms was performed; as well as the isolation of contaminant microorganisms for what the techniques of membrane filtration was used. For the identification of the more representative species there were made conventional biochemical tests and quick systems: API . The results show that water serving as tap water for purification systems fulfill with the microbiological requirements to this kind of water. All the isolated microorganisms were gramnegative bacterias characteristics of this environment: *Pseudomonas putida*, *Xanthomonas maltophilia*, *Aeromonas salmonicida ssp. salmonicida*, *Flavimonas coryzihabitans* and *Acinetobacter iwoffi*. The ultrafiltration and distillation tested systems fulfill with the established microbiological limits, except for deionization and distilled water storing systems. The isolation showed that approximately the 76.9% were of Gram-negative bacterias, the 14.6% of Gram-positive cocci and the 8.5% Gram-positive sporulated bacilli. The most representative genus of purified water were: *Pseudomonas*, with the higher percent of incidence, *Staphylococcus*, *Bacillus*, *Flavobacterium*, *Sphingomonas*, *Aeromonas* and *Agrobacterium*
Key words: *contaminant bacteria, water.*

RESUMEN. Diferentes sistemas de agua que son empleados en la Industria Biofarmacéutica se caracterizaron desde el punto de vista bacteriológico. Para ello se realizó el conteo de microorganismos aerobios mesófilos; el aislamiento de los microorganismos contaminantes, con el empleo de la técnica de filtración por membrana y la identificación de las especies más representativas, a través de pruebas bioquímicas convencionales y sistemas rápidos API. Los resultados muestran que las aguas que sirven como fuente de abasto a sistemas de purificación, cumplen con los requerimientos microbiológicos para aguas potables. Todos los microorganismos aislados fueron bacterias Gram negativas características de este ambiente: *Pseudomonas putida*, *Xanthomonas maltophilia*, *Aeromonas salmonicida ssp. salmonicida*, *Flavimonas coryzihabitans* y *Acinetobacter iwoffi*. Los sistemas de ultrafiltración y destilación estudiados cumplieron con los límites microbiológicos establecidos, no así los sistemas de desionización, ni las cubetas de almacenamiento del agua destilada. La caracterización aportó que el 76.9% de los aislamientos correspondió a bacterias Gram negativas, el 14.6 % a cocos Gram positivos y el 8.5% a bacilos esporulados Gram positivos. Los géneros más representativos de aguas purificadas resultaron ser: *Pseudomonas*, con el mayor porciento de incidencia, *Staphylococcus*, *Bacillus*, *Flavobacterium*, *Sphingomonas*, *Aeromonas* y *Agrobacterium*.

INTRODUCTION

Water plays a fundamental role within the Pharmaceutical Industry being the principal solvent employed. From there the necessity of controlling their microbiological qualities, then the water could constitute the primary source of contamination for pharmaceutical products.[31]

In biotechnological processes, water quality oscillate: from drinking water until purified water and for injection, and they are employed with several objects in function of their qualities.

The methods of established water treatment[1, 29] allow to obtain pure chemically water but not necessarily free of microorganisms. This microorganisms may degrade the product, may contribute endotoxins to the product, by products of microbial metabolism may contaminate the product and finally may be themselves or interact chemically with the product. The purified water qualities depend on the type of treatment and of the method of storage employed.

Although in water could appear Gram-positive and Gram-negative bacterial cells, generally the presence of Gram-positives is a signal of terrestrial run-off or a point source of pollution. The knowledge of the cell type is useful in controlling the contamination of a water system, information about metabolism and growth kinetics of microbial contaminants is also essential.

There are different methods for the identification of microorganisms based on biochemical tests,[5,23] some of them automated[3] and another they have allowed to shorten the

time for the obtaining of results with the development of microtube systems.[11,13,15,18,19]

The present work allows to characterize the water systems used in the Pharmaceutical Industry from the bacteriological point of view, through the counting of mesophilic aerobic microorganisms, as well as the isolation and the identification of the more representative bacteria in drinking and purified waters, applying conventional biochemical tests and quick systems: API.

MATERIALS AND METHODS

Sampling method. The sampling was carried out for a period of 1 year. Two samples of each point were processed weekly, taking in all the cases 500 ml in glass flasks sterile, for the samples of drinking water the flasks contained 0.1 ml of sodium thiosulphate 3% for each 100 ml of water,[1] with the object of inactivate the presence chlorine (approximately 0.2 mg/ L).

All the process was carried out under aseptic conditions.

Samples of 3 points corresponding to tap water of the treatment systems were analyzed; 3 points of ultrafiltration systems, conformed each one for a deionizer, a ultrafilter and a bacteriological filter (0.22μm); 3 points of a deionization system constituted by a carbon bed, an anionic, a cationic and a mixed bed; 2 points of distillation systems of multiple-effect, as well as a corresponding point to the storage bucket of the water once distilled. Each one of these points works from independent form, except the bucket that they are on line with the distillators.

The samples were processed at one time minor of 2 h after picking up.

Counting of the total mesophilic aerobic microorganisms of the samples. Considering the limits established by the USP XXII[28] for drinking water (500 CFU/ml) and purified (100 CFU/ml), the counting of the total mesophilic aerobic microorganisms was determined: for purified waters: 3 Petri dishes with 1 ml of the sample were inoculated following the pour plate method, the culture media employed was PCA:[1] Standard Plate Count Agar (Oxoid).

For drinking waters: 5 ml of water was filtered to reduced pressure employing Sartorius membranes 0.45 μm. The membranes were placed in Petri dishes containing PCA. The volume of sample to filter was determined for the historic behavior of the counting in our drinking waters.

The plates were incubated 48 hours of 30-32°C determining the number of Colony Forming Units (CFU) presents.

Multiple-tube fermentation technique for members of the coliform group. The standard test for the coliform was carried out by the multiple-tube fermentation technique through the presumptive-confirmed phases, results were reported in terms of the Most Probable Number (MPN) of organisms present.[1]

In the presumptive phase five fermentation tubes with an inverted vial in the interior each one were arranged, then 10 ml of Lactose Broth (Oxoid) prepared to double concentration were added. The liquid into the each tube covered the inverted vial. Each tube was inoculated with 10 ml of sample and the test portions were mixed in the medium by gentle agitation. The incubation was carried out at a temperature of 35 ± 0.5°C. Production of gas in the tubes within 48 ± 3 h constitutes a positive presumptive reaction and then the confirmed phase is carry out, but the absence of gas formation at the end of 48 ± 3 h of incubation constitutes a negative test and the test is finished.

Microorganisms isolation The isolation was carried out through the technique of membrane filtration.[1] A 100 ml sample was passed in triplicate through a 0.45 μm membrane filter to reduced pressure for purified water and for drinking water was passed 5 ml of sample.

Once retired the membranes of the team of filtration, it was placed on a nutrient pad impregnate with an specific culture media: Standard, Cetrimide and Endo, for each filtered sample.

All the process was carried out under aseptic conditions.

For total microorganisms counting, the incubation was carried out at 30 to 32°C for 48 h (Standard), the remainder of the nutrients pads was incubated at 35 to 37°C for the same period of time.

The more representative colonies were selected and described their morphologic-cultural characteristics[21] for their hind identification.

Figure 1 shows the diagram of work followed for the microorganisms isolation.

Identification. The method described in the Cowan and Steel's Identification Manual[5] was followed in order to locate each microorganism within a genus or group of genera with conventional biochemical tests, for this the colonies previously selected were isolated again in PCA and were incubated at 30 to 32°C during 18 to 24 h with the object of getting pure and physiologically adequate cultures.

Subsequently it was determined: morphology, Gram reaction, spores production, motility, aerobic and anaerobic growth, catalase and oxidase production, acid production and oxidation-fermentation of carbohydrates (Fig. 2).

The relationship with the Oxygen was demonstrated from Thioglycolate Medium U.S.P. (Oxoid), distributed in tubes at a reason of 20 ml. Once inoculated the medium was incubated at 30 to 32°C for 24h.[21,27]

In order to demonstrate the acid production it was added glucose at a final concentration of 1% (w/v) in Broth Red Phenol (Biocen).[21]

For the tests of oxidation-fermentation glucose was added at a final concentration of 1% (w/v) to a media base conformed by: Peptone 0.2% (w/v), NaCl 0.5% (w/v), K_2HPO_4 0.03% (w/v), Bacteriological Agar 0.5% (w/v),

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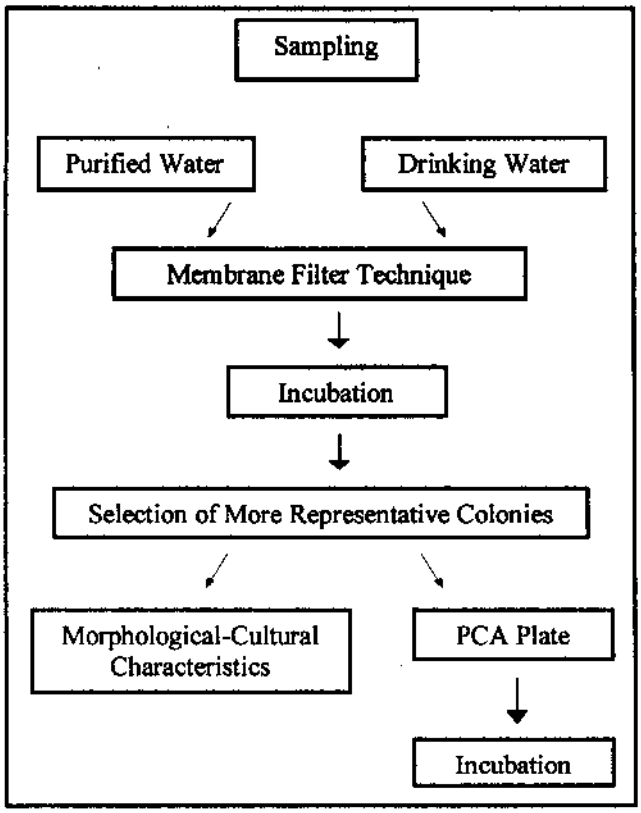

Fig. 1. Diagram of the procedure followed in the isolation of the more representatives bacteria of the analysed water systems.

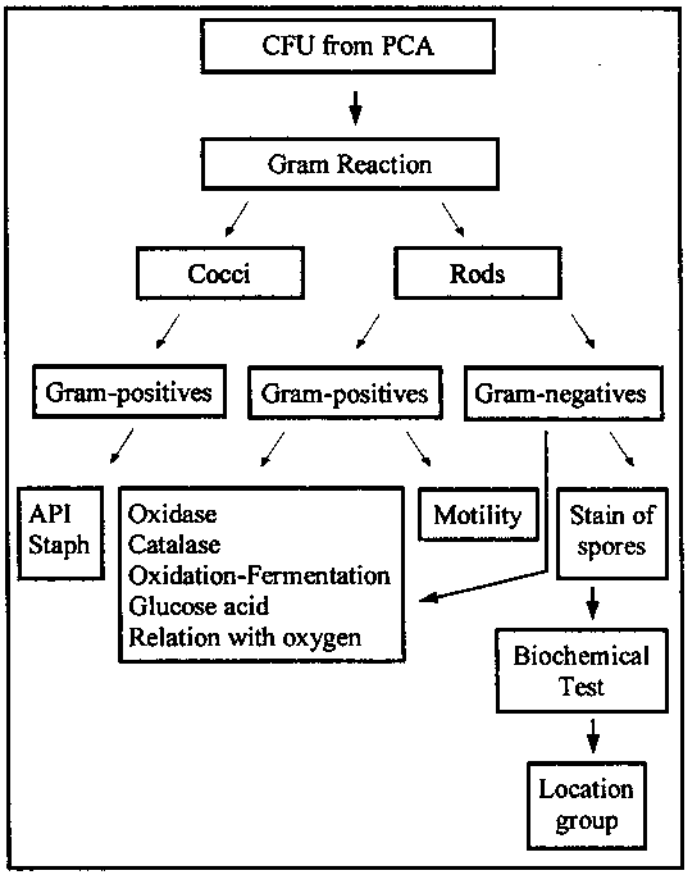

Fig. 2. Diagram of consecutive procedure in order to be located to the microorganisms in a genus or in a genera group.

0.3 ml of a Blue Bromothimol solution 1% (w/v) and pH = 7.1.[21]

The medium was sterilized by autoclaving at 115°C for 20 min..

Gram-negative bacteria identification system. Figure 3 shows the procedure followed in the identification up to species of microorganisms corresponding to Enterobacteria or not Enterobacteria, for which the API 20E[24, 25, 32] and API 20NE systems (bioMérieux)[10, 26] were used. Getting the results in 24-48 h.

Gram-positive cocci identification system. The Gram-positive cocci species that have been located within the *Staphylococcus* and *Micrococcus* generous for biochemical tests, was determined with the application of the API Staph system (bioMérieux).[12, 26]

Endospore-forming Gram-positive rods identification system. To define the species corresponding to the *Bacillus* genus it was proceeded with the following biochemical tests: formation of chains of cells, position and form of the spore, growth at 50°C, growth in NaCl at 10%, acid production from cellobiose, galactose, mannose, melibiose, raffinose, salicine and xylose, OPNG, citrate utilization, urease production, indole production from tryptophan, acetyl-methyl-carbinol production from glucose (Voges

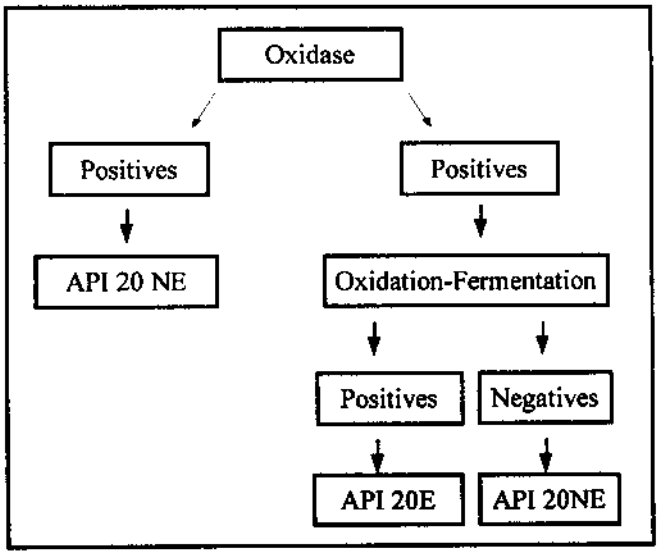

Figure 3. Procedure followed in the identification of Gram-negative rods.

Proskauer reaction), reduction of nitrates to nitrites, casein and starch hydrolysis.[5, 24]

RESULTS AND DISCUSSION

Tap water could become into contamination exogenous source of the treatment systems, because that microorganism could grow in certain grade due to the quantity of organic substances and minerals that are in the solution, therefore the microbiological analysis of these sources constitutes an essential step for the control of the purified waters.

The mesophilic aerobic microorganisms counting (Fig. 4) shows that these waters fulfill the established limits (500 CFU/ml),[29] observing no tendency in function with the season attending on the increase or decrease of the present contaminations. The multiple-tube fermentation technique demonstrated gas absence in the Durham tubes in the presumptive test, then the probability of contamination with coliform microorganisms results very little, less of 2.2 MPN of total coliform microorganisms/100 ml of tested water.[1] These aspects are close related with the execution of the chlorination established cycles of water.

The microorganisms isolated from drinking water points (Table 1) were Gram-negative bacteria considerate typical of the aquatic environments.[5, 30] Although the species of *Aeromonas* and *Acinetobacter* isolated could act like opportunistic pathogens.[5]

In ultrafiltered and distilled waters the mesophilic aerobic microorganisms counting (Fig. 5 and 6) stayed, in general, within the established limits (100 CFU/ ml)[29] This phenomenon didn't behave the same in the deionization systems (Fig. 7), neither in the bucket employed in order to

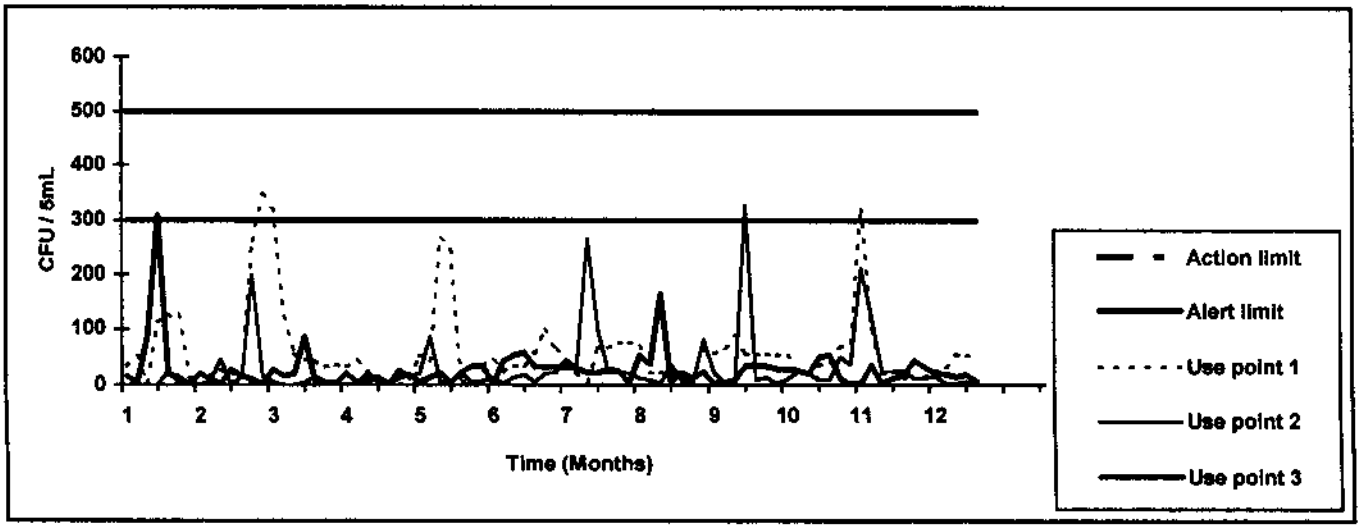

Fig. 4. Microbiological behavior of the drinking water employed like tap water. Mesophilic aerobic microorganisms counted during a year.

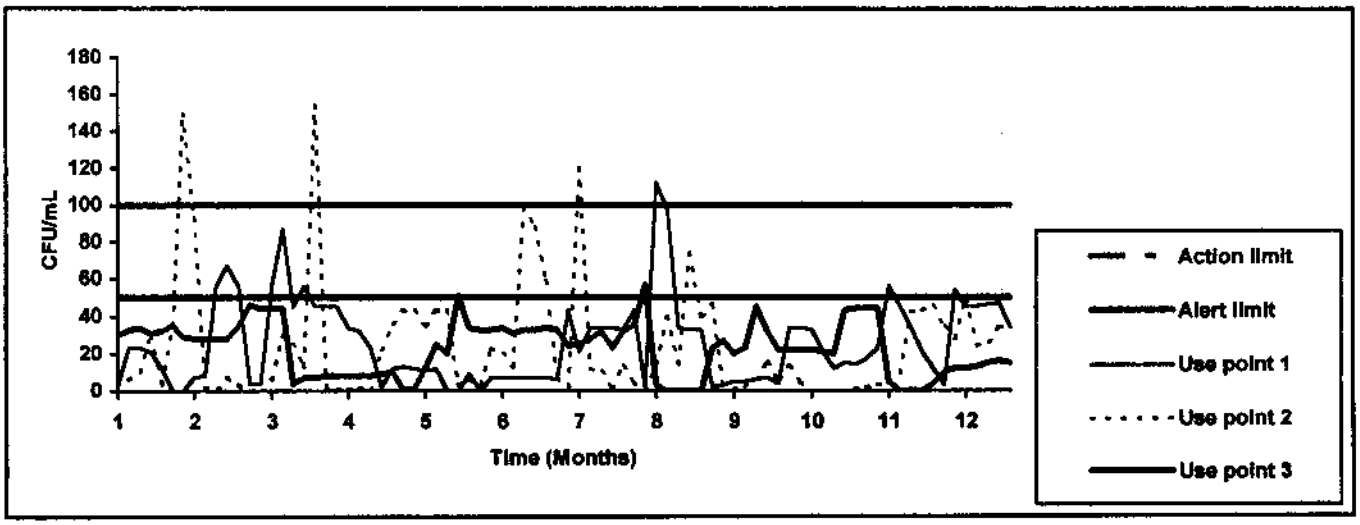

Fig. 6. Microbiological behavior of the ultrafilter water.

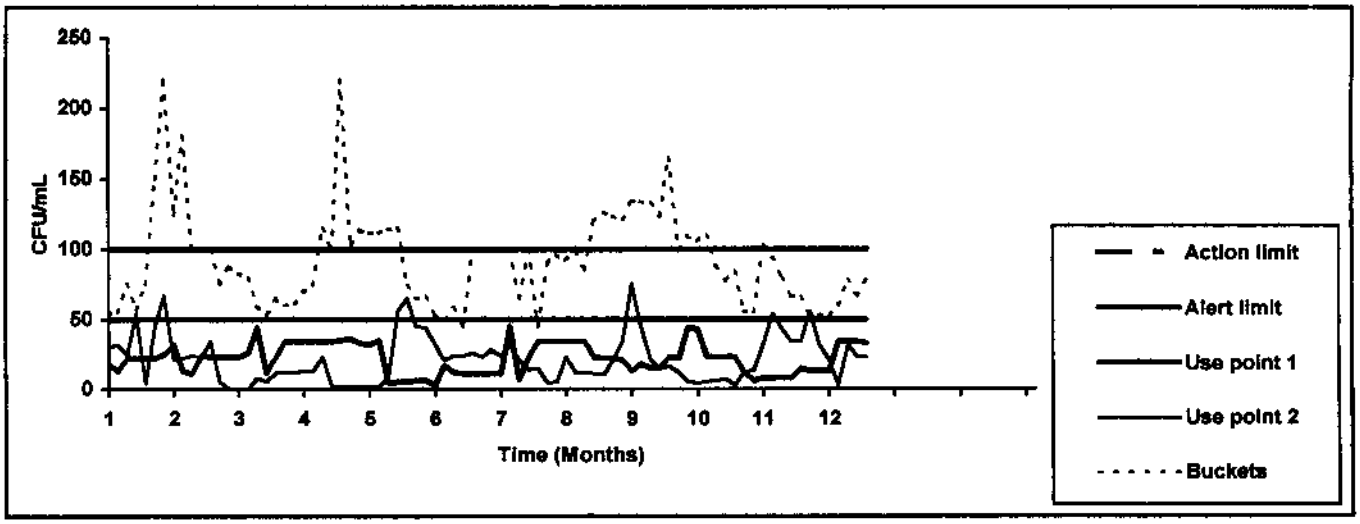

Fig. 6. Microbiological behavior corresponding to the distilled water and the storage bucktets. Mesophilic aerobic microorganisms count during a year.

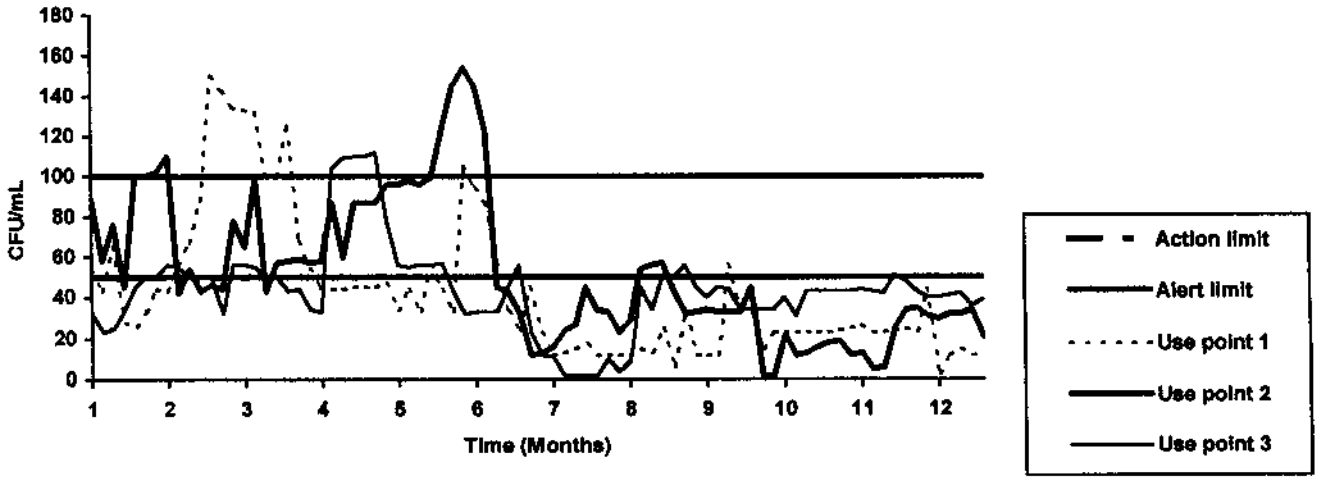

Fig. 7. Microbiological behavior of the deionized water. Mesophilic aerobic microorganisms count during a year.

store the distilled water (Fig 6). In the first case there were influence of the design problems of the facilities that were not built with sanitary pipings and that they have no additives (ultraviolet lamps, ozone generators) that allows to eliminate the microorganisms present in the water.[8] Nevertheless filters were placed of 0.22 μm in the points of use, which although improved the microbiological results, according to Figure 7 (since the month of June), it is not the appropriated, since the cartridges of filters are damaged so rapidly and there are microorganisms that could be able to pass the membranes of the filters.[7]

The design problems in the storage system of the distilled water are evident: water should stay in a distribution loop in which static accumulations of water does not exist and where the temperature control can be possible between 80-85° C. [8] The system of storage in bucket are inadequate, because the formation of a biofilm is provided.[7]

According to Table 2, in the purified waters by ultrafiltration, approximately the 34% of the isolated bacteria by the filtration technique for membrane belong to the *Pseudomonas* genus, with 8 opposing species: *P. picketti* and *P. cepacia* with the higher incidence. This genus meets conformed by heterotrophic Gram-negative bacteria, motile that possesses a great nutritional versatility and capacity of degrade great quantity of organic substances.[5,14]

In falling order, between the principal isolated genera, it met: *Staphylococcus*, represented by 6 species that contributed in a 10.6% of incidence and *Flavobacterium* that was detected in a 9.2 %, resulting *F. indologenes* the only founding species.

Three different species of *Micrococcus* were also found and represented the 7.6%, the *Chrysomonas* and *Coma-*

Table 1. Isolated microorganisms from drinking water employed like tap waters. Fundamental biochemical tests.

Gram	B(-)	B(-)	B(-)	B(-)	B(-)
Oxidase	+	-	-	+	-
CatalaseE	+	+	+	+	+
O/F	-	O	-	F	-
Acid from glucose	-	+	-	+	-
Motility	+	+	+	-	-
Aerobic growth	+	+	+	+	+
Anaerobic growth	-	-	-	+	-
API System	20NE	20E	20E	20NE	20E
Microorganism	*Pseudomonas putida*	*Flavimonas coryzihabitants*	*Xanthomonas maltophilia*	*Aeromonas salmonicida ssp. achromogenes*	*Acinetobacter iwoffi*

B (-), Gram-negative rods; O, oxidation; -, negative result; F, fermentation; +, positive result

Table 2. Isolating frequency (%) of the identified microorganisms from water ultrafiltration systems.

MICROORGANISM	%	MICROORGANISM	%	MICROORGANISM	%
Pseudomonas picketti	10.8	*S. hominis*	1.5	*Xanthomonas maltophilia*	4.6
P. cepacia	7.7	*S. epidermidis*	1.5	*Enterobacter sakasaki*	3.1
P. aureofasciens	4.6	*Flavobacterium indologenes*	9.2	*E. aglomerans*	1.5
P. fluorescens	3.1	*Micrococcus sedentarius*	4.6	*Acinetobacter baumannii*	3.1
P. diminuta	3.1	*M. luteus*	1.5	*A. johnsonni/junni*	1.5
P. versicularis	1.5	*M. lylae*	1.5	*Flavimonas coryzihabitants*	1.5
P. stuzeri	1.5	*Chryseomonas luteola*	6.2	*Chromobacterium violaceum*	1.5
P. putida	1.5	*Comamonas testosteroni*	3.1	*Shewanella putrefasciens*	1.5
Staphylococcus capitis	3.1	*C. acidivorans*	3.1	*Alcaligenes xylosoxidans*	1.5
S. xylosus	1.5	*Bacillus polymixa*	1.5	*Ochrobactrum anthropi*	1.5
S. intermedius	1.5	*B. lentus*	1.5		
S. sciuri	1.5	*B. firmus*	1.5		

monas contributed the 6.2% of the total of isolated bacteria while *Xanthomonas, Acinetobacter, Enterobacter* and *Bacillus* conformed the 4.4% of independent form. The rest of the microorganisms constituted Gram-negative bacterias.

In deionized waters (Table 3) appear the same form that in the previous case, primacy of the *Pseudomonas* genus (33.3% of incidence), represented by *P. putida* and *P. fluorescens*. It next the isolated species was *S. cohnii* (22.2% of incidence), consecutive for other microorganisms that resulted Gram-negative bacterias.

The distillation is the purification water system recommended by the USP,[31] but the microbiological analysis of the distilled water (Table 4) reported a 25% of isolations of *Pseudomonas*, due to the presence of *P. cepacia, P. picketti* and *P. pseudoalcaligenes*.

Bacillus was identified in a 14.4 % and presence of *Flavobacterium, Staphylococcus* and *Sphingomonas* was confirmed in a 10.7 %.

The remainder of the microorganisms evidenced in the piping of leaving from the distillator are in their majority Gram-negative rods.

Table 5 shows a prevalence of not fermenting-glucose

Table 3. Isolating frequency (%) of the identified microorganisms from water deionization systems.

MICROORGANISM	%	MICROORGANISM	%	MICROORGANISM	%
Pseudomonas putida	22.2	*Flavobacterium indologenes*	11.1	*Chryseomonas luteola*	11.1
P. fluorescens	11.1	*Acinetobacter johsonni*	11.1	*Aeromonas salmonicida*	11.1
Staphylococcus cohnni	22.2				

Table 4. Isolating frequency (%) of the identified microorganisms from water distillation systems.

MICROORGANISM	%	MICROORGANISM	%	MICROORGANISM	%
Pseudomonas cepacia	14.3	*B. brevis*	3.6	*Enterobacter cloacae*	7.1
P. picketti	3.6	*Flavobacterium indologenes*	10.7	*Erwinia nigrifluens*	7.1
P. pseudoalcaligenes	7.1	*Sphingomonas paucimobilis*	10.7	*Xanthomonas maltophilia*	7.1
Bacillus pumillus	3.6	*Staphylococcus lentus*	3.6	*Micrococcus sedentarius*	3.6
B. firmus	3.6	*S. cohnni*	3.6	*Acinetobacter baumannii*	3.6
B. circulans	3.6	*S. epidermidis*	3.6		

Table 5. Isolating frequency (%) of the identified microorganisms from storage system of distilled water by buckets.

MICROORGANISM	%	MICROORGANISM	%	MICROORGANISM	%
Pseudomonas cepacia	10.3	*Agrobacterium radiobacter*	17.2	*A. salmonicida ssp. achromogenes*	3.4
P. pseudoalcaligenes	3.4	*Bacillus popillae*	6.9	*Staphylococcus ludunersis*	3.4
P. aureofasciens	3.4	*B. insolitus*	6.9	*S. lentus*	3.4
Sphingomonas paucimobilis	13.8	*Aeromonas caviae*	6.9	*Micrococcus sedentarius*	3.4
S. multivorum	3.4	*A. salmonicida ssp. salmonicida*	3.4	*Enterobacter cloacae*	10.3

Gram-negative rods, especially *Pseudomonas* and *Sphingomonas*, who they only differ from the content of sphingolipids in the wall of the last genus; *Agrobacterium* was also found in the same proportion, this genus comprises primarily pathogens of plants.[5] From like form that these 3 genera contributed equitably the 51.6% of the isolations carried out in total; while *Bacillus* and *Aeromonas* showed a 13.8% of incidence respectively.

The obtained results concordant with all the isolated pollutants have in common the matter of being heterotrophic microorganisms capable of employing varied sources of Carbon, in nutrient poor environments and are capable of showing a variety of strategies in order to grow and survive:[2,4,6,7,16,17] a high surface area / volume ratio, high affinity nutrient transport, chemotaxis, low turn over of cell components, optimization of replication, dormancy and attachment.

Gram-negatives represent the dominant population in the purified water (Fig. 8). The structure of the two different cell types, Gram-negatives and Gram-positives, offers some insight into the probable advantage possessed by the Gram-negatives: The Gram-negative cell has an inner-cytoplasmic membrane on which rests a single layer of peptide glycan and protein; an outer membrane then surrounds these layers and a periplasmic space and is connected to the inner membrane by lipoproteins. The Gram-negative envelope, in addition of being much less rigid than the Gram-positive, is functional more complex. It is more conceivable that the Gram-negative bacterium more efficiently scavenges, binds, or translocates the few useful solutes in a nutrient poor environment than does a Gram-positive cell. Alternatively, the Gram-negative envelope might be advantageous in a survival mode where metabolism is essentially shut down. The very effective barrier provided by outer membrane may serve as a useful protective function.[20]

Nevertheless is important to know that a high concentration of Gram-negatives in tried waters could increase the

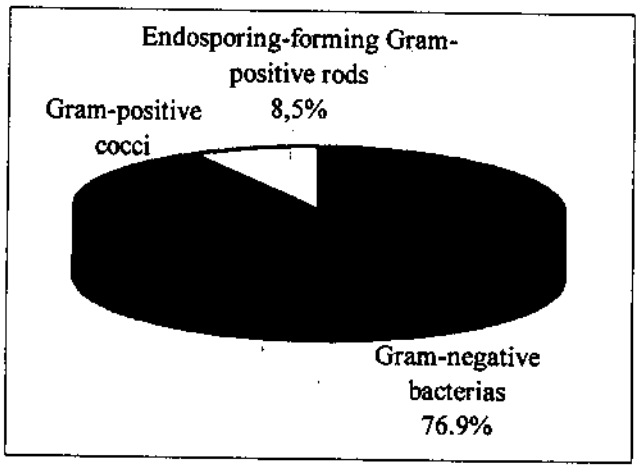

Fig. 8. Frecuency in porcent of the different isolated microbial groups in purified water.

endotoxins level, contribute to the degradation of the product and change the biochemical properties in the same.

The presence of Gram-positives is not common in purified waters, the frequency in which they appear the *Staphylococcus* and *Micrococcus* genera, in the analyzed waters could be due to errors in the sampling, then these microorganisms which are considered environmental pollutants, have little probabilities of proliferate in nutrient poor environments.[20] Although the same phenomenon could have propitiated the presence of *Bacillus*, the microorganisms that conform this genus can be able to contaminate the purified waters and the systems of waters.[19]

In the case of distilled waters, it is necessary to mark that the probability that this contamination should be produced in the interior of the distillator or during the process is very low, that's why is considered the obtained results could be due to the formation of a biofilm in the output piping from the distillator.

The biofilm has a protective effect to microorganisms. Bacteria in the film may survive the addition of sanitizing agents that are lethal to free-floating organisms. In fact, the biofilm constitute a contamination source in the purified water systems (Carbon beds, membrane filters and other surfaces) and in the distribution water systems (valves and pipings).[7]

On the other hand, sanitation in the storage of the distilled water in bucket is difficult from the point of practical view, then the microbiological proliferation is feasible. The circulating loops, as well as the storage of water at 80°C are important factors to considerate in order to achieve low counts of microorganisms and pyrogens, since prevent or slow the attachment and therefore the formation of biofilm.

Another aspect to consider can be the employ of hoses that remain in contact with dirty surfaces or that don't keep subjected to adequate sanitation cycles, propitiating the contamination in the water storage systems.

In all the analyzed systems the *Pseudomonas* genus have prevailed, it is considered highly resistant to the sanitizing agents, still completing the cycles of sanitation of hoses, pipings and teams of treatment of water.

Other genera were also identified with high frequency: *Staphylococcus* which is considered environmental pollutant and not of the water, for this reason should be carried to an extreme the measurements in order to avoid the maximum contaminations in the sampling; *Bacillus*, that is a genus with all their endospore-forming species and can be able of surviving in aquatic environments;[5] *Sphingomonas, Flavobacterium, Aeromonas* and *Agrobacterium*, that are Gram-negative bacterias with possibilities to proliferate in water.[5]

In spite of the presence of microbiological contaminants in several systems of treatment of waters, the microorganisms counting could be reduced applying determined measured: Firstly, attack a strict microbiological control of the tap waters and their chloration once a week,[1] sanitizing the hoses regularly with NaOH at 10%, as well as apply the procedures of sanitation to the systems of ultrafiltration and deionization established by the manufacturers could be profitable. It is important that the water treatment systems are conformed by valves and sanitary pipings and that have coupled in line additions that reduce the bioburden. The cycles of regeneration of ionics beds and the cycles of sanitation of the Carbon beds with vapor or hot water could be effective. Also the water storage should be carried out at temperatures of 80 to 85°C in sanitary tanks or in circulating loops.[8]

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