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Survival of *Escherichia coli* and *Aeromonas hydrophila* in Non-Carbonated Mineral Water

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A b s t r a c t

The study was carried out on the survival of *Escherichia coli* and *Aeromonas hydrophila* in samples of mineral waters. Enumeration of the bacteria was performed by spread inoculation of samples (0.1 cm^3) over the surface of selected media in Petri plates. Twenty bottles (four bottles of each of the five brands) of non-carbonated mineral waters with different levels of dissolved solids and organic content were chosen to study every strain. Ten bottles were stored at 4°C, the other ten were kept at 22°C. Half of the samples of mineral water was filtered, the remaining water was unfiltered. The resulting growth curves depended on the time of storage. The number of *E. coli* increased during the first two weeks (except the seventh day) and decreased during the next days. *E. coli* was detected in 70% of samples of water after 182 days. The number of *A. hydrophila* decreased during the first three days, increased on the seventh day and decreased during the next days. *A. hydrophila* was detected in 15% of the samples of water after 182 days. The temperature of storage was inessential for growth. The most important factors were the brand and the filtering or unfiltering of water. The highest numbers of the bacteria analysed were detected in filtered water, irrespective of the water brand and temperature of storage.

K e y w o r d s: mineral water, *Escherichia coli*, *Aeromonas hydrophila*, survival

Introduction

In recent years, there has been a considerable increase in consumption of bottled water in the world, and this trend is expected to continue. Moreover, uncarbonated water is now considerably more popular than carbonated, having become a substitute for tap water in some households. This reflects consumer concerns about tap water, since bottled water is often regarded as safer and healthier than tap water.

According to the European Community Directive (EC, 1998) and Polish standards (Regulation of the Minister of Health, 2003), natural mineral water is not sterilised, pasteurised or otherwise treated to remove or destroy microorganisms. The number of bacteria recovered at the source is generally very low, around 10–100 cfu cm^{-3}, but there are many reports that viable counts increase, notably in uncarbonated water stored at 25°C, to 10^4–10^5 cfu cm^{-3} after 1–3 weeks of storage (Mavridou, 1992; Mavridou *et al*., 1994; Tsai and Yu, 1997) or longer (Armas and Sutherland, 1999). Allochthonous bacteria, which are contaminants, usually enter water during bottling. Some studies have shown that populations of these bacteria increase to approximately 10^3–10^6 cfu cm^{-3} after bottling (Hunter, 1993). Their survival in bottled water is generally poor because of a low nutrient concentration in the water. However, mineral water has been a cause of outbreaks of waterborne diseases and pathogenic bacteria and viruses have been isolated from mineral uncarbonated water after several weeks of storage (Biziagos *et al*., 1988).

Pathogenic bacteria like *E. coli* were reported to have survived and multiplied in bottled water, which could potentially threaten the health of consumers (Kerr *et al*., 1999). Tsai and Yu (1997) found that *E. coli* grew quickly in autoclaved mineral water from an initial count of 10^2 cfu cm^{-3} to the maximum count of 10^5 cfu cm^{-3} and this count was constant for at least 25 days. Some opportunistic pathogenic bacteria, such as *Aeromonas hydrophila*, have long been known to be an opportunistic pathogen to humans, causing wound infections, sepsis and gastroenteritis (Merino *et al*., 1995). Those organisms have frequently been observed

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in surface water (Kersters *et al*., 1995), drinking water or even bottled water (Massa *et al*., 2001; Warburton *et al*., 1998). Because drinking untreated water could be identified as a significant risk factor for *Escherichia coli* and *Aeromonas hydrophila* associated diseases (Szewzyk *et al*., 2000; Kirow, 1997), it is very important to investigate the survival of these bacteria in the water during storage at different temperatures.

Experimental

Materials and Methods

Bottled water. Eight bottles (1.5 dm^3), all with the same expiry date, of each of the five brands of mineral non-carbonated bottled waters in polyethyleneterephthalate bottles (PET) with different levels of dissolved solids and organic content (Table I) were purchased directly from manufacturers. Half the samples of mineral water, filtered through sterile membrane filters (0.22 μm-pores size Millipore) to remove autochthonous microorganisms, were poured to sterile PET bottles (disinfected with ethanol and rinsed sterile demineralized water), the remaining samples of water were unfiltered.

Table I
Analytical characteristics of five brands of bottled water (information on label of bottle – mg dm^{-3})

Brand	pH	Total mineral components	HCO_3^-	SO_4^{2-}	Cl^-	F^-	SiO_2	Ca^{2+}	Mg^{2+}	Na^+	K^+
N	8.0	700	483.6	–	7.1	0.24	32	111.9	23.3	12.5	5.1
Ż	6.5	185.84	109	–	4.6	0.07	–	27.73	8.18	8	–
B	6.2	–	–	<180	<180	–	–	75–95	35–45	<20	–
M	7.4	644.5	439.3	31.6	5.3	–	–	121.4	21.4	3.3	1.3
T	7.4	263	159.87	10	7.09	0.19	–	43.61	5.83	8	1.6

Sampling. Genetically-marked *Escherichia coli* and *Aeromonas hydrophila* isolated from potable water in our laboratory were used in this study. *E. coli* was inoculated into samples of mineral waters at a density of 2×10^4 cfu cm^{-3} and the inoculation of *A. hydrophila* was performed at a density 1.6×10^4 cfu. The density of final suspension used for inoculation was measured with quantitative methods on the surface of selected media (viable counts). Four bottles of each brand of water (two bottles of filtered water and two bottles of unfiltered water) were inoculated with *E. coli*; the other four bottles of each brand of water (two filtered and two unfiltered water) were inoculated with *A. hydrophila*. Two bottles (one bottle of filtered water and one bottle of unfiltered water) of each type of water inoculated with *E. coli* and two bottles (one bottle of filtered water and one bottle of unfiltered water) of each type of water inoculated with *A. hydrophila* were stored for six months (182 days) at 4°C, the other samples were stored at 22°C. Viable counting of tested bacteria was repeated after 1, 3, 7, 14, 28 and 182 days of storage.

Microbiological analyses. Total viable count of heterotrophic bacteria at 22 and 37°C in 1 cm^3 (pour plate technique) and presence of *E. coli* and *A. hydrophila* in 250 cm^3 (membrane filter technique) of each sample of raw mineral water were tested first. Microbiological analyses involved determination of: 1. count of *Escherichia coli* bacteria in 1 cm^3 of inoculated water on m-FC media (Merck) after 24 hours of incubation at 44.5°C; 2. count of *Aeromonas hydrophila* bacteria in 1 cm^3 of inoculated water on mA agar (Rippey, Cabelli, 1979) after 24 and 48 hours of incubation at 37°C.

Microbial numbers were estimated by decimal dilution in Ł strength Ringer's solution. The water samples were inoculated on the surface of selected media in Petri plates and incubated at a specified temperature for the desired period of time. After the incubation typical colonies were counted. To determine the number of *E. coli* bacteria, blue coloured colonies were counted. For the determination of the number of *A. hydrophila* bacteria, yellow coloured colonies were counted.

If no colonies were recovered with the surface plating technique, pour plates with 1cm^3 of water sample were prepared with the same selective media.

Statistical evaluation. In order to obtain information concerning potential differences between bacteria numbers for various brands of water, for filtered/unfiltered water, for water stored at different time and temperature, a single factor analysis of variance (ANOVA) was conducted, verifying the hypothesis of the equality of means ($H_0: x_1 = x_2 = \ldots = x_5$) at the level of significance $\alpha = 0.05$, assuming that the variance for the numerousness of the bacteria groups under study are uniform. The uniformity of variance was tested with Levene's test. If the test proved significant, the hypothesis was rejected. Next, the Kruskal-Wallis test was applied, which is a non-parametric equivalent of the analysis of variance (Stanisz, 1998).

Results and Discussion

Bacterial numbers. The numbers of *E. coli* and *A. hydrophila* in 250 cm^3 of raw mineral water were unidentifiable. Total viable count of heterotrophic bacteria at 22 and 37°C in 1 cm^3 of raw mineral water was the highest for brands T and M and the lowest for brand Ż (detailed results can be obtained from the authors).

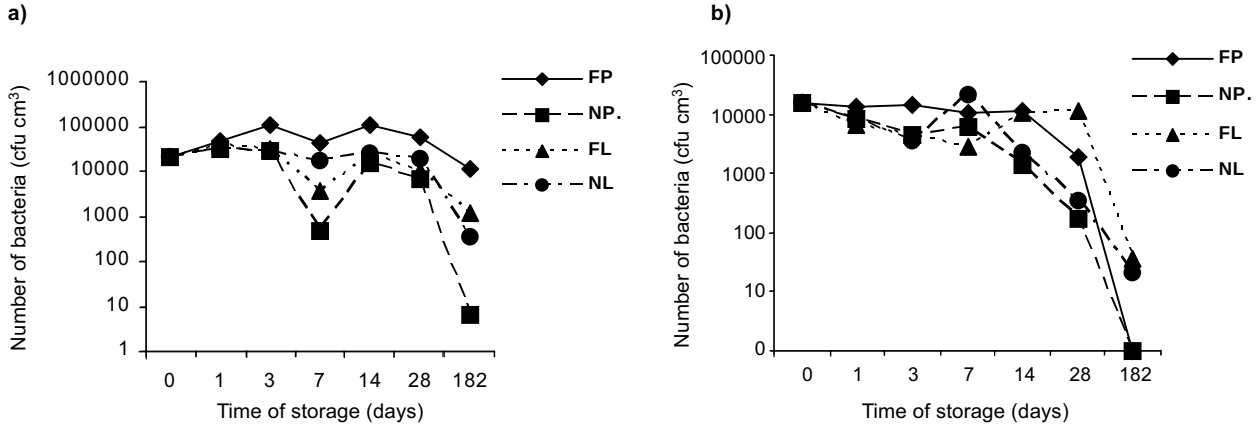

Fig.1. Survival of a) *Escherichia coli* and b) *Aeromonas hydrophila* in filtered and unfiltered non-carbonated mineral water during half-year storage in 4 and 22°C. FP – filtered water stored at 22°C; NP – unfiltered water stored at 22°C; FL – filtered water stored at 4°C; NL – unfiltered water stored at 4°C.

The viable counts of *E. coli* and *A. hydrophila* inoculated into bottled mineral water decreased at both temperatures of storage (4 and 22°C). Both *E. coli* and *A. hydrophila* were typically more numerous in filtered samples of water stored at room temperature (about 22°C) and in unfiltered samples stored at low temperatures (about 4°C) (Figure 1).

Moreira *et al*. (1994), who investigated survival of *E. coli* in still mineral water, reported that decrease in culturability of these bacteria depended on the type of water and the presence or absence of normal flora of mineral water. Ramalho *et al*. (2001) implied that autochthonous bacteria of mineral water have been reported to have an inhibitory effect on the survival of *E. coli* and other pathogenic bacteria. Some authors (Kersters *et al*., 1996) reported that *A. hydrophila* experienced a very strong first order logarithmic decline after inoculation in unfiltered water, but was capable of surviving in filtered-autoclaved freshwater. Rippey *et al*. (1994) found that for *A. hydrophila* densities of 10^5 cfu cm^{-3} were attained in filtered-autoclaved freshwaters. These densities were three to eight orders of magnitude higher than those occurring in natural environments. The removal of existing microbiota from nutrient poor waters significantly affects the survival capacity of *A. hydrophila*. Relatively low densities of these bacteria found in unfiltered water are a result of population control by external factors, which may inhibit bacterial population.

In our experiment the number of *E. coli* increased during the first two weeks (except the seventh day) and decreased during the next days (Figure 2). The bacteria were detected in 70% of water samples after 182 days of storage. The number of *A. hydrophila* decreased during the first three days, increased on the seventh day and decreased during the next days (Figure 2). *A. hydrophila* was detected in 15% of water samples after 182 days of storage.

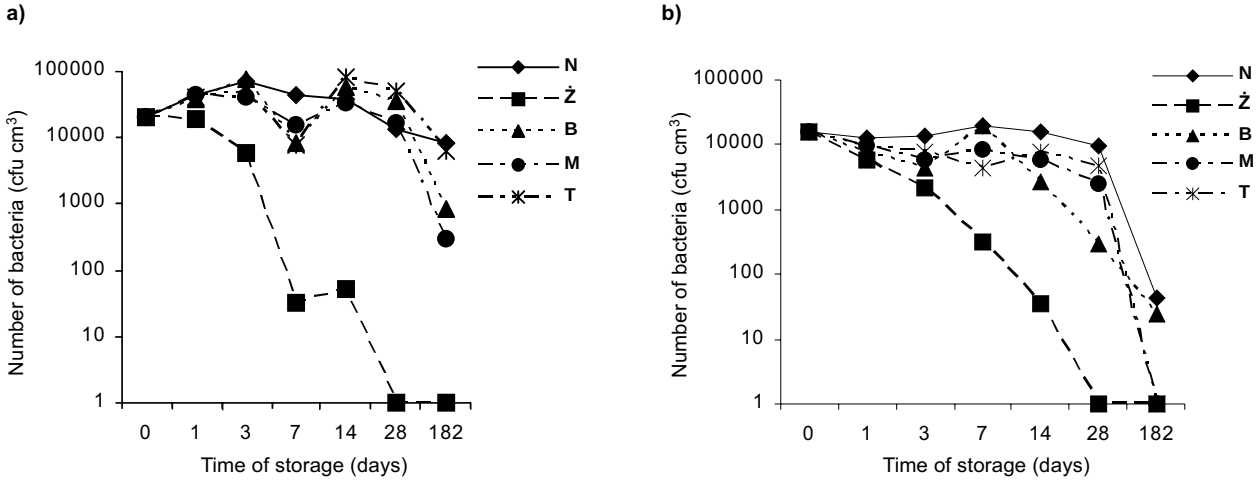

Fig. 2. Survival of a) *Escherichia coli* and b) *Aeromonas hydrophila* in different brands of non-carbonated mineral water (as in Table I) during half-year storage after inoculation.

The survival of the bacteria examined in mineral water was the lowest in Ż mineral water, which contained the lowest levels of dissolved solids and organic content. Both types of bacteria inoculated into this brand of water were unidentifiable after 28 days of storage. Their highest values were usually recorded in mineral water with higher level of dissolved solids and organic content, notably in N and T brands. Bernagozzi *et al*. (1995) noticed that the number of *A. hydrophila* in water was affected by the quantity of nutrients present in water.

The survival of *E. coli* in uncarbonated mineral water was higher than *A. hydrophila*. Enumeration of *E. coli* and *A. hydrophila* recovered from water decreased respectively to 10^3 and 10^1 cfu 1 cm^3 after 182 days of storage. For comparison, Tsai and Yu (1997) ascertained that the count of *E. coli* was two orders of magnitude higher than that of *A. hydrophila* in autoclaved uncarbonated mineral water after 35 days of storage at 25°C.

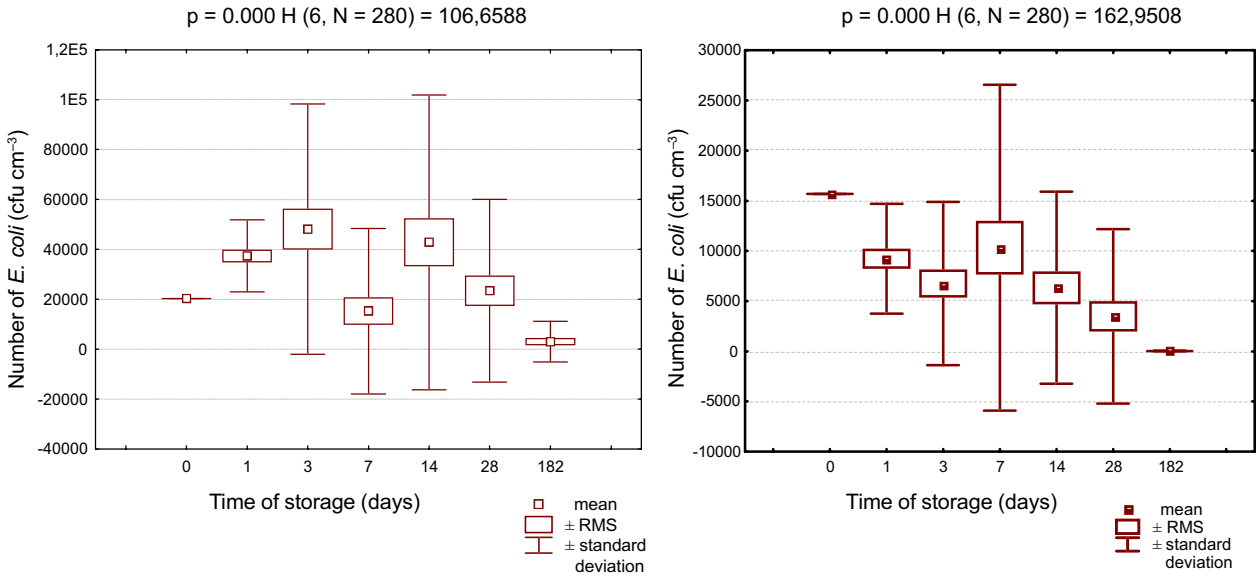

Fig. 3. Averages numbers (± standard deviation and ± random mean square-RMS) of *E. coli* and *A. hydrophila* inoculated into mineral water during storage-total viable counts (cfu cm^{-3}). Independent variable (assembling): time. ANOVA test of Kruskala-Wallisa ranges.

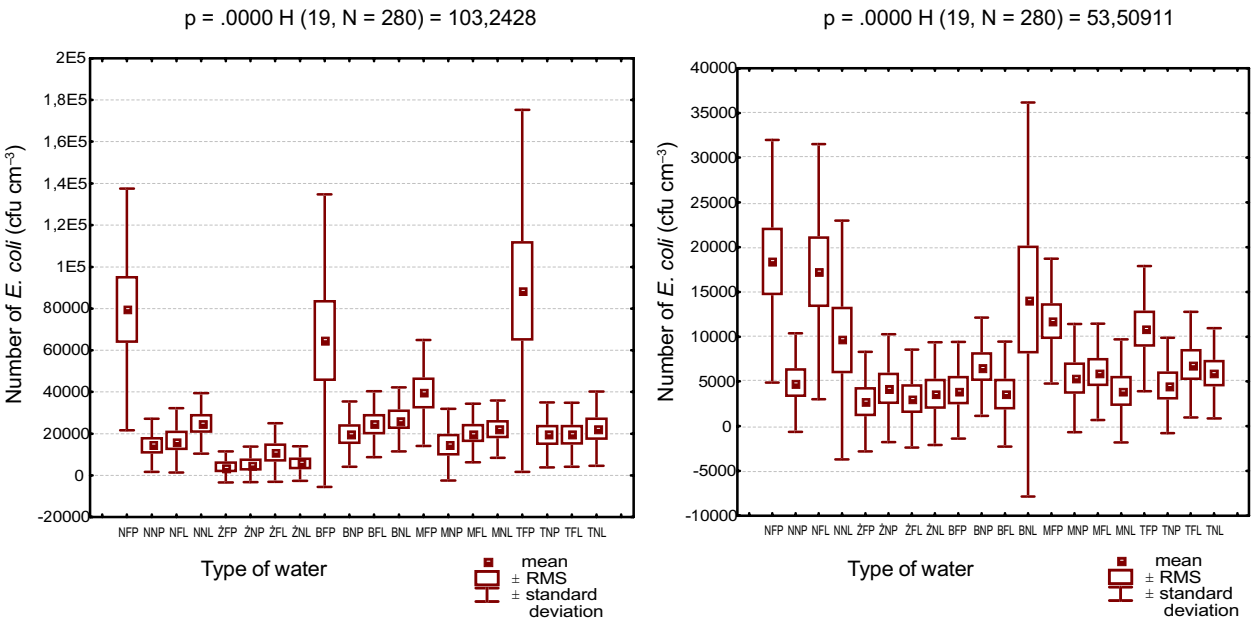

Fig. 4. Averages numbers (± standard deviation and ± random mean square-RMS) of *E. coli* and *A. hydrophila* inoculated into filtered/unfiltered mineral water during storage at 4 and 22°C – total viable counts (cfu cm^{-3}). Independent variable (assembling): type of water (brand+temperature of storage+filtration/unfiltration). ANOVA test of Kruskala-Wallisa ranges. First letters N, Ż, M, B and T – mean brand of water; second letter – F, N – mean filtered/unfiltered water; third letters – L, P – mean temperatures of storage – 4/22°C.

Statistical evaluation. This paper presents only the general statistical relationships; the detailed results can be obtained from the authors.

There were significant differences between the numbers of *E. coli* and *A. hydrophila* recovered from the different brands of bottled water (respectively $p = 0.0000$ and $p = 0.0001$). The mean numerousness of the tested bacteria was the highest in N and T brands.

There were significant differences between the numbers of tested microorganisms initially presented in the water and those after storage for 6 months (for both microorganisms $p = 0.000$) (Figure 3).

There were significant differences between the numbers of studied microorganisms recovered from the filtered and unfiltered water (respectively $p = 0.001$ and $p = 0.04$). The mean numbers of the tested bacteria were the highest in filtered water stored at temperatures 22°C and in unfiltered water stored at temperatures 4°C (Figure 4).

There were no significant differences between the numbers of tested microorganisms recovered from the water stored at temperatures 4 and 22°C.

Conclusions

The growth of *E. coli* and *A. hydrophila* inoculated into bottled mineral water depended on the time of storage and decreased during storage. The kind of brand and the filtering or unfiltering of water were the most important factors for the growth of microorganisms. The survival rate of both bacteria in mineral water was the lowest in water which contained the lowest level of dissolved solids and organic content. Highest rates of survival were usually recorded in mineral water with higher amounts of dissolved solids and organic matter. The most numerous bacteria were detected in filtered water, irrespective of the water brand or temperature of storage. Higher counts of the bacteria were typically observed in filtered samples stored at room temperature (about 22°C) and in unfiltered samples stored at low temperatures (about 4°C). The survival of *E. coli* in non-carbonated mineral water was higher than that of *A. hydrophila*. Because mineral water can be identified as a significant risk factor for *Escherichia coli* and *Aeromonas hydrophila* associated diseases, survival of these bacteria in water during storage at different temperatures is a very important research question.

Literature

Armas A.B. and J.P. Sutherland. 1999. A survey of the microbiological quality of bottled water sold in the UK and changes occurring during storage. *Int. J. Food Microbiol*. **48**: 59–65.

Bernagozzi M., F. Bianucci and R. Sacchetti. 1995. Prevalence of *Aeromonas spp.* in surface waters. *Water Environ. Res*. **67**: 1060–1064.

Biziagos E., J. Passagot, J.M. Crance and R. Deloince. 1988. Long-Term of Hepatitis A Virus and Poliovirus Type 1 in Mineral Water. *Appl. Environ. Microbiol*. **11**: 2705–2710.

EC. 1998. Council Directive relating to the quality of water intended for human consumption, 98/83/EEC. OFF. *J. Community.* L330, 32–54.

Hunter P.R. 1993. A review: The microbiology of bottled natural mineral waters. *J. Appl. Bacteriol*. **74**: 345–352.

Kerr M., M. Fitzgerald, J.J. Sheridan, D.A. McDowell and I.S. Blair. 1999. Survival of *Escherichia coli* O157:H7 in bottled natural mineral water. *J. Appl. Microbiol*. **87**: 833–841.

Kersters I., L. Van Vooren, G. Huys, P. Janssen, K. Kersters and W. Verstraete. 1995. Influence of temperature and process technology on the occurrence of *Aeromonas* species and hygienic indicator organisms in drinking water production plants. *Microb. Ecol*. **30**: 203–218.

Kersters I., G. Huys, H. Van Duffel, M. Vancanneyt, K. Kersters and W. Verstraete. 1996. Survival potential of *Aeromonas hydrophila* in freshwaters and nutrient-poor waters in comparison with other bacteria. *J. Appl. Bacteriol*. **80**: 266–276.

Kirow S.M. 1997. *Aeromonas* and *Plesiomonas* species. In: Doyle M.P., L.R.Beuchat and T.J. Montville (eds), Food Microbiology. Fundamentals and Frontiers, ASM Press, Washington DC, USA, 265–287.

Massa S., C. Altieri and A. D'Angela. 2001. The occurrence of *Aeromonas* spp. in natural mineral water and well water. *Int. J. Food Microbiol*. **63**: 169–173.

Mavridou A. 1992. Study of the bacterial flora of a non-carbonated natural mineral water. *J. Appl. Bacteriol*. **73**: 355–361.

Mavridou A., M. Papapetropoulou, P. Boufa, M. Lambiri and J.A. Papadakis. 1994. Microbiological quality of bottled water in Greece. *Lett. Appl. Microbiol*. **19**: 213–216.

Merino S., X. Rubires, S. Knřchel and J.M. Tomàs. 1995. Emerging pathogens: *Aeromonas* spp. *Int. J. Food Microbiol*. **28**: 157–168.

Moreira L., P. Agostinho, P. Vasconcellos Morais and M.S. da Costa. 1994. Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass. *J. Appl. Bacteriol*. **77**: 334–349.

Ramalho R., A. Alfonso, J. Cunha, P. Teixeira and P.A. Gibbs. 2001. Survival characteristics of pathogens inoculated into bottled mineral water. *Food Control*. **12**: 311–316.

Regulation of the Ministry of Health. 2003. Official Gazette, 89/ 842 from 18th April 2003.

Rippey S.R. and V.J. Cabelli. 1979. Membrane filter procedure for enumeration of *Aeromonas hydrophila* in fresh waters. *Appl. Environ. Microbiol*. **38**: 108–113.

Rippey S.R., M.A. Troy and V.J. Cabelli. 1994. Growth kinetics of *Aeromonas hydrophila* in freshwaters supplemented with various organic and inorganic nutrients. *World J. Microbiol. Biotechnol*. **10**: 159–164.

Stanisz A. 1998. Przystępny kurs statystyki w oparciu o program STATISTICA PL na przykładach z medycyny, StatSoft Poland Sp. z o.o., Kraków, 263–292.

Szewzyk U., R. Szewzyk, W. Manz and K.H. Schleifer. 2000. Microbiological safety of drinking water. *Annu. Rev. Microbiol*. **54**: 81–127.

Tsai G.J. and S.C. Yu. 1997. Microbiological evaluation of bottled uncarbonated mineral water in Taiwan. *Int. J. Food Microbiol*. **37**: 137–143.

Warburton D., B. Harrison, C. Crawford, R. Foster, C. Fox, L. Gour and P. Krol. 1998. A further review of the microbiological quality of bottled water sold in Canada: 1992–1997 survey results. *Int. J. Food Microbiol*. **39**: 221–226.