Food Control 33 (2013) 63–67

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Diachronic long-term surveillance of bacteriological quality of bottled water in Greece (1995–2010)

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ARTICLE INFO

Article history:
Received 22 August 2012
Received in revised form
15 January 2013
Accepted 23 January 2013

Keywords:
Bottled water
Diachronic surveillance
Bacteriological quality
Greece

ABSTRACT

The aim of this study is the diachronic surveillance (1995–2010) of the microbiological quality of non-carbonated bottled water sold in Greece. One thousand eight hundred and sixty samples, derived from 29 bottling companies (22 domestic and 7 imported) have been analyzed. The samples were analyzed, using standard methods (ISO) for the detection and enumeration of Total coliforms, *Escherichia coli*, *Enterococcus* spp., *Pseudomonas aeruginosa* and Heterotrophic plate count at 22 °C and 37 °C. 13.0% of the tested samples were characterized as non-comply with human consumption according to Greek legislation, due to the presence of Total coliforms, *P. aeruginosa* or *Enterococcus* spp. These microorganisms detected in 9.1%, 6.1% and 1.0% of the samples respectively. The study shows a significant improvement of the quality of the bottled water over time. Also, there was significant difference of microbiological burden among specific brand names and not geographic areas. The present study is one of the very few long-term studies evaluating quality of bottled water in Greece. The study shows a diachronic presence of indicators in bottle water and may help the bottling companies to improve the water quality. The diachronic detection of the bacterial burden in bottled waters highlights the need for systematic and rigorous controls, both in the bottling and during storage and maintenance on the market.

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1. Introduction

The global bottled water industries increase their sales year by year. The global bottled water market grew by 4.7% in 2009 to reach a value of $79,844.6 million. Europe accounts for 50.9% of the global bottled water market value (Datamonitor, 2010). The fact that the consumption of bottled water has increased over the last years, may reflect the public's concern for the water quality of tap water and in parallel their consideration that bottled water is microbiologically and chemically safer than tap water. Moreover, it is considered to be more complying with drinking, especially for immunocompromised patients and infants (Khaniki et al., 2010; Venieri, Vantarakis, Komninou, & Papapetropoulou, 2005).

According to the European Directives 2009/54/EC and 98/83 EEC adapted by Greek legislation, it should be noted that a bottle water sample is considered non-complying with human consumption when it is not in accordance to at least one parameter of the ones mentioned in directives that it is to be free of microorganisms such as Total coliforms, *Escherichia coli*, faecal Streptococci and *Pseudomonas aeruginosa*. Also, the heterotrophic plate count (HPC) should not exceed 100 CFU (colony forming units) per ml at 22 °C for 72 h and 20 CFU per ml at 37 °C for 24 h, after 12 h of bottling.

Several studies have demonstrated the detection of microbes in bottled water. These products usually failed to comply with the established national and international standards and they were not suitable for human consumption (Hussein, Hassan, & Bakr, 2009; Iwersen et al., 2009; Khaniki et al., 2010; Venieri et al., 2005; Zeenat, Hatha, Viola, & Vipra, 2009). Heterotrophic bacteria, such as *Pseudomonas* spp., *Klebsiella*, *Aeromonas hydrophila*, *Vibrio cholerae*, *Enterococcus* spp. etc, isolated from water samples, showed also multi-drug resistance (Allen, Edberg, & Reasoner, 2004; Kokkinakis, Fragkiadakis, & Kokkinaki, 2008; Zeenat et al., 2009). This is of major importance concerning public health. *E. coli* and other faecal coliforms, Enterococci and Enterobacteriaceae belong to the traditional indicators of faecal pollution, whereas *P. aeruginosa* is indicated for wound infections, neonatal and nosocomial infections, such us tracheobronchitis and pneumonia. The growth of bacteria, of human or animal origin, is favored by short period and high temperature incubation. In contrast, the longer incubation at low temperature indicates the growth of water-based autochthonous microorganisms (Allen et al., 2004).

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0956-7135/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2013.01.034

In Greece, although the bottling industry is in significant growth in the last decade, only few studies concerning the microbiological quality as well as the consumption of bottled waters have been performed (Kokkinakis et al., 2008; Mavridou, Papapetropoulou, Boufa, Lambiri, & Papadakis, 1994; Venieri et al., 2005). The aim of the present study was a diachronic evaluation of the bacteriological quality of bottled water (natural mineral and bottled table water) sold in Greece, during the period 1995–2010.

2. Materials and methods

2.1. Sampling

From 1995 until 2010, a total of one thousand eight hundred and sixty six (1860) samples of non-carbonated (still) bottled natural water, sealed in PET (polyethyleneeterephthalate) bottles, were selected randomly in a monthly base, directly from the shelves of large supermarkets in Patras. Samples were comprised of 1754 domestic (22 brands) and 106 imported ones (7 brands from France and Italy). Samples were immediately transferred to the laboratory and stored at 4–8 °C until their analysis the same or the next day.

2.2. Bacteriological analysis

All the samples were tested with ISO standard methods for the detection of Total coliforms (ISO 9308-1:2000), *E. coli* (ISO 9308-1:2000), *Enterococcus* spp. (ISO 7899-02:2000), *P. aeruginosa* (ISO 16266:2006), Heterotrophic bacteria (ISO 6222:1999). The results were compared to the reference criteria contained in Directive 2009/54/EC of the European Parliament and the Council of the European Union (2009) on the exploitation and marketing of natural mineral waters (Table 1).

2.3. Statistical analysis

The microbiological results were evaluated using IBM SPSS 20.0 and analysis of variance (ANOVA). Statistical comparisons between geographic areas and brand names were performed. The results were compared to the reference criteria contained in European Directives on the exploitation and marketing of natural mineral waters.

3. Results and discussion

In total, 1860 samples of non-carbonated mineral (68.9%) and table water (31.1%) were analyzed for the presence and enumeration of Total coliforms, *E. coli*, *Enterococcus* spp., *P. aeruginosa*, Heterotrophic plate count at 22 °C and 37 °C. The samples were randomly sampled from supermarket stores in Greece during the period 1995–2010. The samples' characteristics data are shown in Table 2. The samples were seasonally collected as follows: autumn 23.5%, winter 14.2%, spring 28.9% and summer 33.3%.

As non-complying sample was considered any sample found positive for the presence of any of the following: Total coliforms, *E. coli*, *Enterococcus* spp. and *P. aeruginosa*. The 13.0% of the tested samples were considered as samples that are not complying with human consumption. Analytically, Total coliforms were detected in 170 samples (9.1%). However, none of the analyzed samples tested positive for *E. coli*. *Enterococcus* spp. was detected in 18 samples (1.2%) and *P. aeruginosa* was detected in 114 out of 1860 samples (6.1%). Among all the brands, brands 12, 13, 14, 19, 20, 21, 27 and 28 (8/29) did not show any non-compliance. Statistical data for each brand are shown in Table 2. Brand 22 had the higher percentage of positive samples for Total coliforms and *Enterococcus* spp. (8%). Furthermore, *Enterococcus* spp. was detected in brands 6, 8, 22 and 25. As it concerns *P. aeruginosa*, brand 24 had the higher percentage of positive samples (Table 2). Fig. 1 presents a map with the (%) of samples of domestic brands in each area that not complying with the guidelines. There were some brands with significant higher presence of positive samples, although there was no correlation of the positive samples with the geographic area ($p < 0.005$).

Similarly to our study, the presence of Total coliforms in bottled water has previously been reported. Varga (2011) indicated the presence of Total coliforms, *E. coli*, *Enterococcus* spp. and *P. aeruginosa* in 6.3%, 1.4%, 1.2% and 1.4% of the 492 bottled water samples sold in Hungary. Also, our results agree to Semerjian's study (2011), where 18.8% of samples were positive for Total coliforms in bottled water sold in Lebanon. According to the results of Zamberlan da Silva et al. (2008), Total coliforms detected in 40.2% of 77 samples of bottled water in dispensers, *E. coli* in 6.4% of the samples, *Enterococcus* spp. in 9% and *P. aeruginosa* in 58.4% of the samples. As opposed to these findings, in Kokkinakis et al. (2008) study, none of sixty tested bottled water was positive for indicator pathogens. In this case, the products derived from a unique bottling company, which had implemented HACCP (Hazard Analysis and Critical Control Points) methodology.

Total coliforms do not always correlate with faecal contamination, since the total coliform group includes both faecal and environmental species, they consists more valuable indicator for monitoring water quality than an index of faecal pathogens (WHO, 2008). On the other hand, enteric microorganisms such as *E. coli* and faecal streptococci such us *Enterococcus* spp. demonstrate faecal contamination of human or animal origin (Bharath et al., 2003; Jeena, Deepa, Mujeeb Rahiman, Shanthi, & Hatha, 2006; Venieri et al., 2005). The presence of potent pathogens, such as *P. aeruginosa* in our analyzed bottled samples, in agreement with current literature, shows that bottled water is not safe at the desired level. This oligotrophic bacterium constitutes a perfect indicator for the hygienic quality of drinking water, in particular as it concerns the bottling process (Iwersen et al., 2009; Jeena et al., 2006; Varga, 2011; Venieri et al., 2005). The presence of high numbers of *P. aeruginosa* in bottled water may be associated with complaints about taste, odor and turbidity (WHO, 2008). Despite the fact that *P. aeruginosa* is capable of causing infection in immunocompromized patients, so far, there have not been any cases recorded due to the consumption of bottled water (Fok, 2005; Varga, 2011). Fig. 2 shows the diachronic percentage (%) of detection of each microorganism by year. Total coliforms and *P. aeruginosa* were detected in samples until 2010. In contrast, *Enterococcus* spp. was detectable in some samples until 2000. There is significant improvement of the quality of the bottled waters over time in the most of the brands ($p < 0.005$). Although, the study demonstrates that the continued presence of microbiological load in bottled waters, throughout all these years, highlights the need of inclusion

Table 1
Current microbiological limits for bottled natural mineral waters as established in Directive 2009/54/EC of the European Parliament and the Council of the European Union (2009).

Parameter	Parametric value
Total coliforms	Non-detectable (ND)/250 ml
Escherichia coli	ND/250 ml
Enterococcus spp.	ND/250 ml
Pseudomonas aeruginosa	ND/250 ml
Spore-forming sulfite-reducing anaerobes (Clostridia)	ND/50 ml
Heterotrophic plate count at 20–22 °C[a]	$\leq 1.0 \times 10^2$ CFU/ml
Heterotrophic plate count at 37 °C[a]	$\leq 2.0 \times 10^1$ CFU/ml
Parasites and pathogenic microorganisms	Absent (in full product volume)

[a] To be measured within 12 h after bottling.

Table 2
The characteristics and statistics data of bottled samples from 29 brands, during the period 1995–2010.

Brand code	Type of water	Origin	No (%) of samples tested	No (%) of uncomply with samples	No (%) of samples positive for		
					TC	EN	PA
1	A	D	158 (8,5)	8 (5.1)	6 (3.8)	0 (0.0)	4 (2.5)
2	A	D	282 (15,2)	34 (12.1)	11 (3.9)	0 (0.0)	22 (7.8)
3	A	D	51 (2,7)	4 (7.8)	2 (3.9)	0 (0.0)	2 (3.9)
4	A	D	55 (3,1)	3 (5.2)	1 (1.7)	0 (0.0)	2 (3.4)
5	A	D	51 (2,8)	5 (9.6)	3 (5.8)	0 (0.0)	1 (1.9)
6	B	D	187 (7,40	15 (10.9)	14 (10.1)	7 (5.1)	9 (6.5)
7	B	D	126 (10,7)	7 (3.5)	6 (3.0)	0 (0.0)	2 (1.0)
8	A	D	82 (4,4)	10 (12.2)	8(9.8)	2 (2.4)	2 (2.4)
9	A	D	47 (2,5)	2 (4.3)	0 (0.0)	0 (0.0)	2 (4.3)
10	A	I	35 (1,9)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)
11	A	I	19 (1,0)	1 (5.3)	0 (0.0)	0 (0.0)	1 (5.3)
12	A	D	10 (0,6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
13	B	D	10 (0,3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
14	A	I	26 (1,4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
15	B	D	21 (1,1)	3 (14.2)	1 (4.8)	0 (0.0)	1 (4.8)
16	B	D	21 (0,8)	3 (21.4)	1 (7.1)	0 (0.0)	1 (7.1)
17	B	I	14 (0,8)	2 (14.3)	1 (7.1)	0 (0.0)	1 (7.1)
18	B	D	41 (2,2)	5 (12.2)	4 (9.8)	0 (0.0)	1 (2.4)
19	A	D	12 (0,6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
20	A	I	12 (0,3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
21	A	I	5 (0,3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
22	A	D	244 (13,5)	90 (35.9)	87 (34.7)	8 (3.2)	30 (12.0)
23	A	D	67 (3,6)	10 (11.9)	2 (3.0)	0 (0.0)	8 (12.0)
24	A	D	82 (4,4)	16 (19.5)	5 (6.1)	0 (0.0)	15 (18.3)
25	B	D	78 (4,2)	8 (10.3)	6 (7.7)	1 (1.3)	1 (1.3)
26	B	D	39 (2,1)	2 (5.1)	0 (0.0)	0 (0.0)	2 (5.1)
27	A	D	10 (0,5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
28	A	D	6(0,3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
29	A,B	I,D	49(2,6)	15 (30.6)	12 (24.5)	0 (0.0)	8 (16.3)
Total			1860 (100.0)	242 (13.0)	170 (9.1)	18 (1.0)	114 (6.1)

A: Natural mineral water, B: Table water, D: Domestic, I: Imported; TC: Total coliforms, EN: *Enterococcus* spp., PA: *Pseudomonas aeruginosa*.

of HACCP in order to produce an improved final product, safer for human consumption.

The source of bottled water has to be protected against each type of contamination which can affect the autochthonous flora and water quality. Protection of drilling and systematic control of water during bottling is necessary to ensure the best water quality as well as safer products. Samples, after distribution, should be stored in dark places at temperatures lower than 4–10 °C. This step is essential for the maintenance of the microbiological quality of bottled products, particularly when plastic containers are used, which provides additional substrate for microbial growth (Evandri, Tucci, & Bolle, 2000; Kokkinakis et al., 2008).

Furthermore, of all the bottled water examined, 1565 samples had heterotrophic plate count (HPC) less than 100 CFU ml^{-1} at 22 °C and 1562 samples had HPC below 20 CFU ml^{-1} at 37 °C. It should be noted that the microbiological analysis did not take place within 12 h after bottling as is required. According to national regulation, total heterotrophic flora should not exceed 100 and 20 colonies forming units per milliliter at 22 °C and 37 °C respectively, using the pour plate method, 12 h after bottling. In Table 3, a high percentage of heterotrophic plate count at 22 °C and 37 °C is reported. Despite the fact that the samples did not comply with the regulation, they can not be considered as non-complying with consumption, because the samples were not analyzed after 12 h of the bottling process and the initial amount of autochthonous bacteria, which were in the bottles, could proliferate and reached the amount of 10^3–10^4 CFU ml^{-1} (Zamberlan da Silva et al., 2008) after several days. HPC is a microbiological parameter, which demonstrates the water quality, and the limit can be different among different countries. The limit of 500 CFU ml^{-1} is called "level of concern" by many authors (Allen et al., 2004). High heterotrophic plate count does not directly constitute a health risk, but can cause deterioration in the organoleptic properties of water, such as taste, odor and dimming (and in this case, the sample is not complying with consumption) (Papapetropoulou & Mavridou, 1995). Also, in some cases, the presence of infectious microorganisms with or without drug resistance has been indicated (Abd El-Salam, Al-Ghitany, & Kassem, 2008). This presence probably poses health risks to consumers, especially to immunocompromized patients (Jeena et al., 2006). In addition, several studies observed that, heterotrophic plate count higher than 1000 CFU are able to inhibit the growth of coliform bacteria on culture media (Allen et al., 2004), which might cause deception during the sample's evaluation. According to this case, it should be taken into account that the presence of total coliforms or *P. aeruginosa* in our samples probably be more frequent, since 182 samples had HPC greater or equal to 1000 CFU ml^{-1} at 22 °C, and 127 samples at 37 °C respectively.

In addition, the natural mineral water samples were found not complying with human consumption at a higher percentage, than the bottled table samples, 15.1 and 9.0% respectively. Groundwater is the main source of bottled mineral water. Natural mineral water can not be sterilized or pasteurized to remove or destroy microorganisms (Mavridou et al., 1994; Venieri et al., 2005). A method, which is used to remove all microorganisms except viruses, is micro-filtration (0.22 mm pore size). However, it has been demonstrated that some bacteria are able to pass through the pore zone (Hahn, 2004; Wang, Hammesa, & Eglia, 2008). On the other hand, table water may not have a specific natural origin. Since table water is not connected to a specific spring it may be produced and bottled anywhere and is produced after distillation, deionisation, reverse osmosis and any other form of treatment for human consumption (IBWA, 2010). According to these facts it should be noted that micro-filtration is not always an efficient method of sterilization.

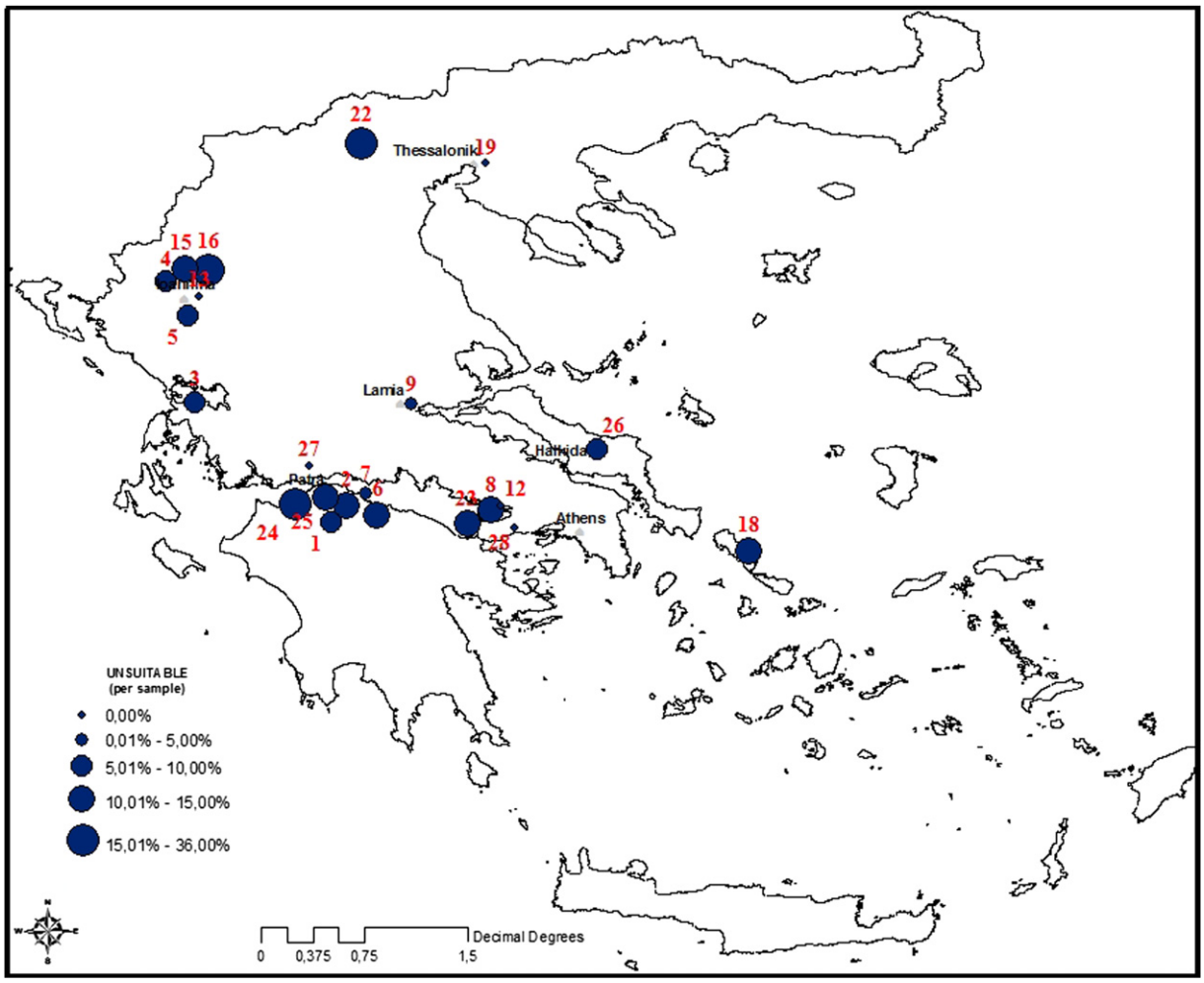

Fig. 1. (%) of non-complying samples of bottling waters per production area.

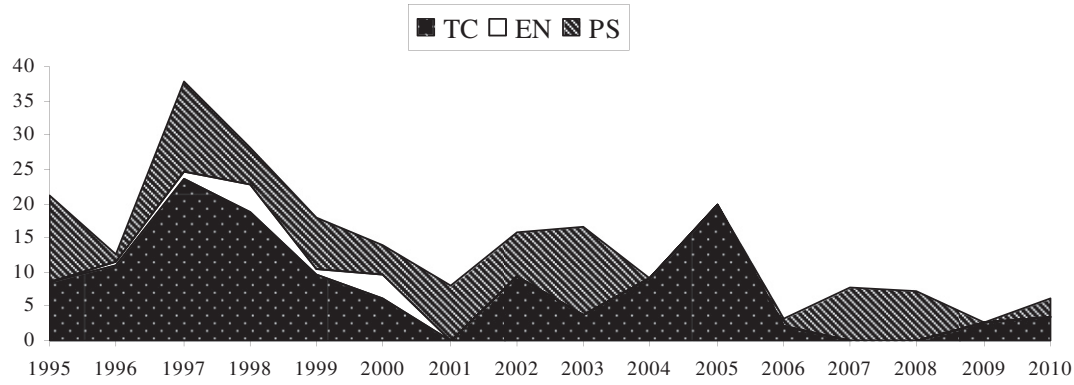

Fig. 2. (%) samples positive for indicator microorganisms' samples during the 1995–2010 period.

Table 3
Heterotrophic plate count (CFU ml^{-1}) of samples tested at 22 °C and 37 °C.

HPC (CFU ml^{-1})	No (%) of samples at	
	22 °C	37 °C
HPC < 20	1476 (79.4%)	1562 (84.0%)
20 ≤ HPC < 100	89 (4.8%)	79 (4.2%)
100 ≤ HPC < 1000	71 (3.8%)	66 (3.5%)
1000 ≥ HPC	182 (9.8%)	127 (6.8%)

4. Conclusion

Our study demonstrates the continued presence of bacteriological burden in bottled water, during the period 1995–2010. The risk of outbreaks of waterborne diseases increases where standards of water, sanitation and personal hygiene are low. Fifteen years represents a long time period and underlines the need for systematic control to achieve top quality products from bottling companies. HACCP guidelines are applied by most of manufacturing bottling companies and supermarkets in order to offer a safe

product from the beginning of bottling until, the distribution and storage of samples at the various points of sale. From the diachronic surveillance of the 29 bottling companies all these years, it is observed that the bacteriological quality of the products is better the last five years than in the period 1995–2000.Unfortunately, this study did not have a consistent distribution of the number of tested samples, either per brand, or year and season because of the inability of Health Officers who performed sampling. It has to be mentioned, that more frequent checks should be imposed by the government and the companies in order to ensure public health.

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