THE INDICATOR PARAMETERS IN MICROBIOLOGICAL ANALYSIS OF DRINKING WATER, INTENDED FOR ANIMAL CONSUMPTION

PARAMETRII-INDICATOR ÎN ANALIZA MICROBIOLOGICĂ A APEI POTABILE, DESTINATĂ ADĂPĂRII ANIMALELOR

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ABSTRACT

In Romania, there isn't any separate standard for animal drinking, considering that water for animal drinking must have the same quality as water for human drinking (potable water). Therefore, the work defines and presents the indicator parameters of microbiological contamination of drinking water and the importance of their analysis in the context of appreciation of quality of drinking water.

There are also presented the methods and working standards for the main microbiological parameters mentioned in European and Romanian legislation are: coliform bacteria, *Escherichia coli*, enterococci, micro-organisms formating colonies at 22°C and 37°C, *Pseudomonas aeruginosa* and *Clostridium perfringens*.

The interpretation of these microbiological parameters is performed according with Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water.

Keywords: drinking water, indicator parameter, microbiological contamination

REZUMAT

În România, nu există un standard separat pentru apa destinată adăpării animalelor, considerându-se că apa destinată adăpării animalelor trebuie să aibă aceeași calitate ca și cea consumată de oameni. Prin urmare, lucrarea definește și descrie parametrii-indicator ai contaminării microbiologice a apei potabile precum și importanța analizării acestora în contextul aprecierii calității apei potabile.

De asemenea, sunt menționate metodele de analiză microbiologică, precum și standardele în care se fac referiri la acestea, pentru principalii parametri microbiologici menționați în legislația europeană și românească sunt: bacteriile coliforme, *Escherichia coli*, enterococii, microorganismele formatoare de colonii la 22°C și 37°C, *Pseudomonas aeruginosa* și *Clostridium perfringens*.

Interpretarea parametrilor microbiologici este efectuată în conformitate cu Legea 311/2004 pentru modificarea și completarea Legii 458/2002 privind calitatea apei potabile.

Cuvinte cheie: apă potabilă, parametru-indicator, contaminare microbiologică

Drinking water is water intended for human consumption. It is represented by any type of water in its original state or after treatment, intended for drinking, cooking, food preparation or other domestic purposes, regardless of its origin and whether it is supplied from a distribution network, from a tanker or in bottles or containers and all waters used as source in food industry for manufacture, processing, preservation or marketing of products or substances intended for human consumption (1, 2).

The scope of this work is to define and present the indicator-parameters of microbiological contamination of drinking water and the importance of their analysis for assessment of drinking water quality.

The indicator-parameters of microbiological contamination of drinking water emphasize the presence of pathogens on basis of pre-existent relationship between the indicator parameter and outbreaking of infection in the population; there are in greater number than pathogens; they don't multiply in environment; they have a comparable resistance to pathogens; they are detected by fast, simple, reliable and cheap techniques (4).

They can be indicators of:

1. Faecal pollution;
2. The effectiveness of water disinfection;
3. Integrity and cleanliness of distribution systems

The main microbiological parameters mentioned in European and Romanian legislation are: coliform bacteria, *Escherichia coli*, enterococci, microorganisms formating colonies at 22°C and 37°C, *Pseudomonas aeruginosa* and *Clostridium perfringens*.

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Coliform bacteria are Gram-negative, aerobic and facultatively anaerobic, non-spore-forming bacilli, capable of growing in the presence of relatively high concentrations of bile salts with the fermentation of lactose. This group include the genera: *Escherichia, Citrobacter, Klebsiella, Enterobacter, Serratia, Hafnia* – species with faecal origin (human and animal faeces) and environmental origin (capable of multiplication in water and soil). They should be absent immediately after disinfection, their presence indicating inadequate treatment, biofilm formation through ingress of foreign material (soil, plants) in distribution systems and water tanks.

Escherichia coli is a member of thermotolerant coliforms group, capable of lactosis fermentation at higher temperature, but it is different from these by: ability to produce indole from triptophan and β-glucuronidase enzyme. It is present in a big quantity in human and animal faeces and very rarely in tropical soils. *Escherichia coli* is considered the **most suitable indicator of faecal contamination**, its presence providing evidence of recent faecal contamination, that imply actions of investigation of potential sources such as: inadequate treatment or breaches in distribution systems integrity.

Enterococci are a subgroup of faecal streptococci (species of *Streptococcus* genus), represented by species:*Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Enterococcus hirae*.They are Gram -positive bacteria, relatively tolerant of sodium chloride (6,5%) and alkaline pH levels, facultatively anaerobic and occur singly, in pairs or as short chains. Enterococci are relatively specific for faecal pollution because most species don't multiply in water environment but some can originate from other habitats, soil, in the absence of faecal contamination. They are considered the **indicators of recent faecal pollution** like *Escherichia coli*, but they tend to survive longer in water environment and are more resistant to drying and chlorination.

Microorganisms formating colonies are represented by bacteria and fungi, capable to grow on rich growth media, without inhibitory or selective agents, over a specified incubation period and at a defined temperature (la 22°C și 37°C). There are included: microorganisms sensitive to disinfection process (coliform bacteria), microorganisms resistant to disinfection (sporulated microorganisms), microorganisms that proliferate rapidly in treated water in absence of residual disinfectants and opportunist pathogens as: *Acinetobacter, Aeromonas, Flavobacterium, Klebsiella, Moraxella, Serratia, Pseudomonas, Xanthomonas*. There is no evidence of an association of any of these organisms with gastrointestinal infection through ingestion of drinking-water in general population.

Microorganisms that grow at 37°C originate from human and animals with warm blood and microorganisms that grow at 22°C are of aquatic origin. Their number should be as low as possible, a big number providing an inadequate treatment, presence of biofilm and lack of integrity of distribution systems.

Pseudomonas aeruginosa is a member of *Enterobacteriaceae* Family, nesporulated, weak mobile, that synthesize pigments like: pyocianin, pyoverdin and pyorubin and is considered a hygiene indicator.

Clostridium perfringens is a species of *Clostridium* Genus, represented by a group of Gram-pozitive, anaerobic, sporulated (spores are resistant in unfavorable conditions of aquatic habitat: UV irradiation temperature and pH extremes and chlorination) sulfite-reducing bacilli. It is considered an **indicator** with high specificity of **intermitent faecal pollution**, an indicator of protozoa and enteric viruses and useful indicator of effectiveness of filtration, because the *C. perfringens* spores are smaller than oocysts or cysts of protozoa.

The methods of analysis of microbiological parameters are mentioned in EU Directive 2015/1787 for modification of EU Directive 98/83/EC on the quality of water intended for human consumption as numbers of european and international method standards (Table 1)

The name of european and international method standards is identified by accessing www.iso.org (11) and introduction of standard number (Tabel 2) and the presence of romanian corresponding standard is detected by accesing www.magazin.asro.ro (12)(Table 3).

The methods of microbiological analysis consist of:

- Sample inoculation by pour plate technique and counting the colonies after incubation (microorganisms formating colonies at 22°C and 37°C);

- Sample filtration (the diameter of pores of membrane filter is 0,45µ), membrane transfer on solid selective medium, couting of presumtive colonies after incubation and their confirmation by biochemical tests or cultivation on/in other solid/liquid selective media (coliform bacteria and *E. coli*, intestinal enterococci, *Pseudomonas aeruginosa*, *Clostridium perfringens*);

- Inoculation of sample and sample dilutions in series of tubes /wells, counting of tubes /wells that present a modification of colour after incubation and determination of most probable number on basis of statistical tables and computer programmes (coliform bacteria and *Escherichia coli*) (7).

Table 1

EU Dir. 2015/1787 for modification of EU Dir. 98/83/EC on the quality of water for human consumption. Part A – Microbiological parameters and methods

Detection and enumeration of *Escherichia coli* and ` coliform bacteria	EN ISO 9308-1 or EN ISO 9308-2
Detection and enumeration of intestinal enterococci	EN ISO 7899-2
Detection and enumeration of *Pseudomonas aeruginosa*	EN ISO 16266
Counting of microorganisms formating colonies at 22°C and 37°C	EN ISO 6222
Detection and enumeration of *Clostridium perfringens*	EN ISO 14189

Table 2

The name of european and international standards

EN ISO 9308-1:2014	Water quality – Detection and anumeration of *Escherichia coli* and coliform bacteria. Part 1: Membrane filtration method for waters with low bacterial background flora
EN ISO 9308-2:2012	Water quality – Detection and anumeration of *Escherichia coli* and coliform bacteria. Part 2: Most probable number method
EN ISO 7899-2:2000	Water quality – Detection and anumeration of intestinal enterococci. Part 2: Membrane filtration method
EN ISO 16266:2008	Water quality – Detection and anumeration of *Pseudomonas aeruginosa* - Method by membrane filtration
EN ISO 6222:1999	Water quality – Enumeration of culturable microorganisms – Colony count by inoculation in a nutrient agar culture medium
EN ISO 14189:2013	Water quality – Detection and anumeration of *Clostridium perfringens* - Method using membrane filtration

Table 3

The correspondence between international, european and romanian standards

EN ISO 9308-1:2014	SR EN ISO 9308-1:2015
EN ISO 9308-2:2012	SR EN ISO 9308-2:2014
EN ISO 7899-2:2000	SR EN ISO 7899-2:2002
EN ISO 16266:2008	SR EN ISO 16266:2008
EN ISO 6222:1999	SR EN ISO 6222:2004
EN ISO 14189:2013	-

The evaluation of number of microorganisms formating colonies at 22°C and 37°C /ml is made by inoculation of 1 ml of sample and sample decimal dilution in Petri dishes, twice, counting of colonies grown on culture medium – yeast extract agar – after aerobic at 22 ± 2°C and 36 ± 2°C and calculation of weighted average (5).

For detection and enumeration of *Escherichia coli* and coliform bacteria, by membrane filtration method: filter 100 ml of samples, transfer membrane on selective medium – chromogenic coliform agar (CCA), with substrates for enzimes as: β-galactozidase (synthesized by coliforms) and β-glucuronidase (synthesized by *Escherichia coli*), incubate the plate at 36±2°C, 21 ±3 h, examine the colonies grown on the plate, considering dark blue-violet - β-galactozidase-positive and β-glucuronidase-positive colonies as *Escherichia coli* confirmed/plate and pink-red colonies - β-galactozidase-positive as coliform bacteria that are not *Escherichia coli* presumtive and their confirmation of the latter by oxidase test (negative) (8).

The principle of the method for detection and enumeration of intestinal enterococci is based on filtration of 100 ml of sample, incubation of membrane on Slanetz-Bartley medium, that contains sodium azide (for inhibition of Gram-negative bacteria) and 2,3,5-triphenyltetrazolium chloride (colourless substance), that is reduced formazan (red) by enterococci. By transfer of membrane with typical colonies on bile-aesculin-azide agar, preheated at 44^{0}C, the hydrolysis occurs in 2 hours (the final product,6,7-dihidroxicumarin, combines with iron (III) ions in order to give a tan-coloured to black compound that diffused into the medium) (6).

The method for detection and enumeration of *Pseudomonas aeruginosa* consists in: filtration of 100 ml of sample by membrane, its transfer on *Pseudomonas* agar base/CN-agar, incubation of plate at 36±2°C, 44±4 h, examination of colonies grown on the plate: blue or green colonies being *Pseudomonas aeruginosa* confirmed colonies and the rest - *Pseudomonas aeruginosa* presumtive colonies, that will be confirmed by: oxidase test, test of ammonia from acetamide and fluorescence test by inculation on King's B medium (9). The detection and enumeration of *Clostridium perfringens* is performed by filtration of 100 ml of sample, membrane transfer on selective medium - tryptose-sulfite-cycloserine agar (TSC), anaerobic incubation at 44±1^{0}C, 21±3 h, examination of colonies grown on the plate, considering black, grey or yellow colonies - *Clostridium perfringens* presumtive colonies (as a result of the reduction of sulfite to sulfide which reacts with ferric salt in the medium) and their confirmation by acid phosphatase test (positive) (10).

Table 4

The scheme of methods for indicator-parameters analysis

Parameter	Detection	Selection	Subculture	Confirmation
Detection and enumeration of *Escherichia coli*	Chromogenic coliforme agar (CCA) 36 ± 2 °C, 21 ± 3 h Presence of dark blue-violet colonies (confirmed)	-	-	-
Detection and enumeration of coliform bacteria	Chromogenic coliforme agar (CCA) 36 ± 2 °C, 21 ± 3 h Presence of pink to red colonies (presumtive)	1-10 colonies/plate (number of colonies/plate? 10) or >10 colonies/plate (number of colonies/plate >10)	+/- Tryptone soy agar (TSA) 36 ± 2 °C, 21 ± 3 h	**Oxidase test** (negative)
Detection and enumeration of intestinal enterococci	Slanetz-Bartley medium 36 ± 2 °C, 44 ± 4 h Presence of pink, red or maroon colonies (presumtive)	-	-	**Test of aesculin hidrolysis** Bile-aesculin-sodium azide agar, 44 ± 0,5 °C, 2 h Presence of black colonies (confirmed)
Detection and enumeration of *Pseudomonas aeruginosa*	*Pseudomonas* agar base/CN-agar 36 ± 2 °C, 44 ± 4 h Presence of blue or green colonies (confirmed)	-	-	-
	Pseudomonas agar base/CN-agar 36 ± 2 °C, 44 ± 4 h Presence of fluorescent colonies not blue or green (presumtive)	+	Nutrient agar 36 ± 2 °C, 21 ± 3 h	**Test of ammonia from acetamide** (positive) Acetamide broth 36 ± 2 °C, 21 ± 3 h The colour of medium modifies from colorless to yellow.
	Pseudomonas agar base/CN-agar 36 ± 2 °C, 44 ± 4 h Presence of reddish-brown colonies (presumtive)	+	Nutrient agar 36 ± 2 °C, 21 ± 3 h	**Oxidase test** (positive) **Test of ammonia from acetamide** (positive) Acetamide broth 36 ± 2 °C, 21 ± 3 h The colour of medium modifies from colorless to yellow. **Fluorescence test** (positive) King's B medium 36 ± 2 °C, 5 days
Counting of microorganisms formating colonies at 22 °C	Yeast extract agar 22 ± 2 °C, 44 ± 4 h Presence of bacterial and fungal colonies	-	-	-
Counting of microorganisms formating colonies at 37 °C	Yeast extract agar 36 ± 2 °C, 68 ± 4 h Presence of bacterial & fungal colonies	-	-	-
Detection and enumeration of *Clostridium perfringens*	Tryptose-sulfite-cycloserine agar (TSC) 44 ± 1 °C, 21 ± 3 h Presence of black, grey or yellow colonies (presumtive)	11-10 colonies/plate (number of colonies/plate? 10) or >10 colonies/plate (number of colonies/plate >10)	Tryptone soy agar (TSA) or agar base 36 ± 2 °C, 21 ± 3 h	**Acid phosphatase test** (positive)

For easy approach of microbiological analysis of drinking water, Tabel 4 can be used, because it presents the scheme of methods for indicator-parameters analysis.

The number of micro-organisms formating colonies at 22°C and 37°C /ml represents the weighted average of numbers of colonies/plates inoculated with sample and dilution 10^{-1} of sample (Fig. 1).

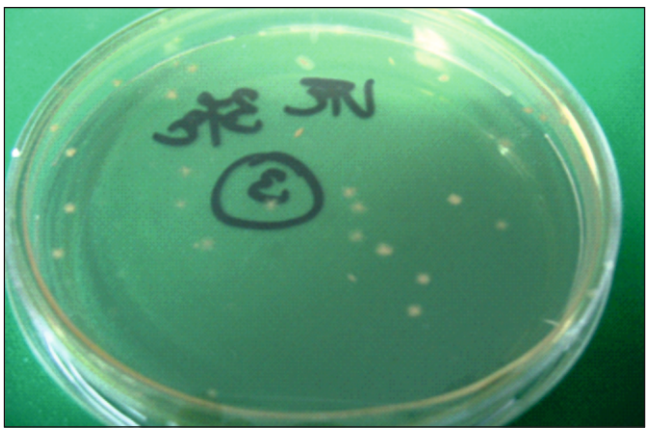

Fig. 1. Colony Count /plate (original)

The number of coliform bacteria /100 ml represents the number of coliform bacteria /plate, expressed as sum between number of *Escherichia coli* confirmed /plate (dark blue-violet colonies - β-galactozidase-positive and β-glucuronidase-positive) and number of coliform bacteria that are not *Escherichia coli* presumtive (pink-red colonies - β-galactozidase-positive) and confirmed (oxidase-negative)/plate (Fig. 2).

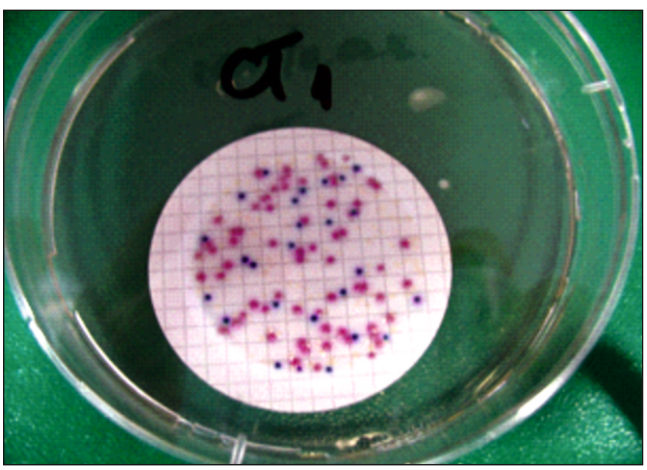

Fig. 2. The presence of *Escherichia coli* (dark blue-violet colonies), coliforms that are not *Escherichia coli* (red colonies) and background (white colonies)/CCA plate (original)

The number of intestinal enterococci /100 ml is the number of presumtive colonies that grow on Slanetz-Bartley medium (pink, red or maroon colonies) (Fig. 3) and confirm on bile-aesculin-azide agar (black colonies, blackening of medium under the colonies) (Fig. 4, 5).

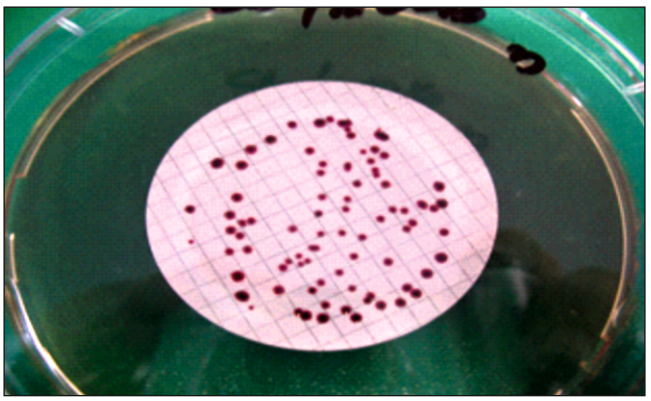

Fig. 3. The presence of presumptive intestinal enterococci (red-brown colonies)/plate with Slanetz-Bartley medium (original)

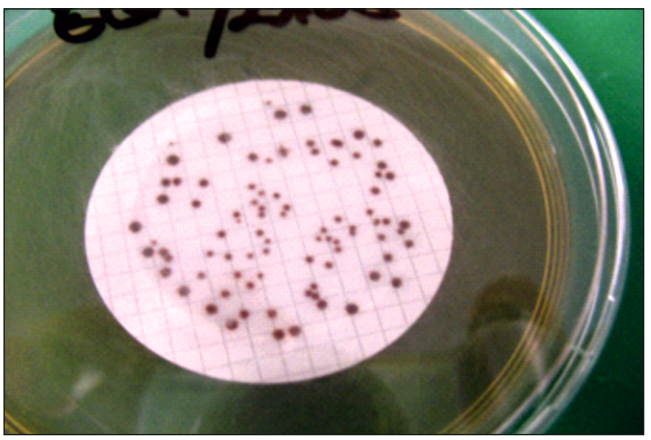

Fig. 4. The presence of confirmed intestinal enterococci (black colonies)/plate with bile-aesculine -sodium azide agar (original)

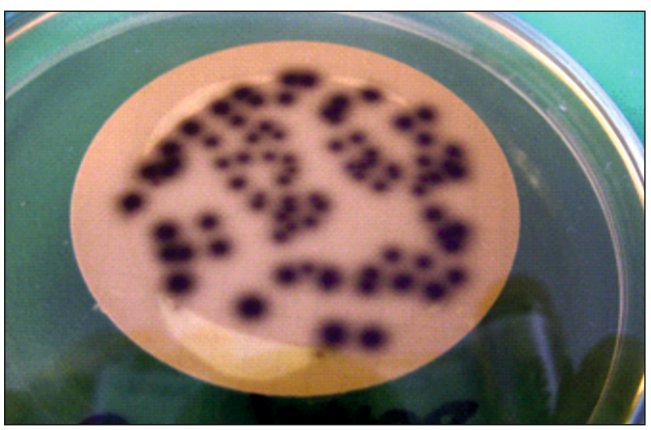

Fig. 5. The presence of confirmed intestinal enterococci (black colonies)/plate with bile-aesculine - sodium azide agar (reverse plate)(original)

The number of *Clostridium perfringens*/100 ml is represented by number of presumptive colonies that grow on tryptose-sulfite-cycloserine agar (black, grey or yellow colonies) (Fig. 6) and confirm by acid fosfatase test (positive) (Fig. 7).

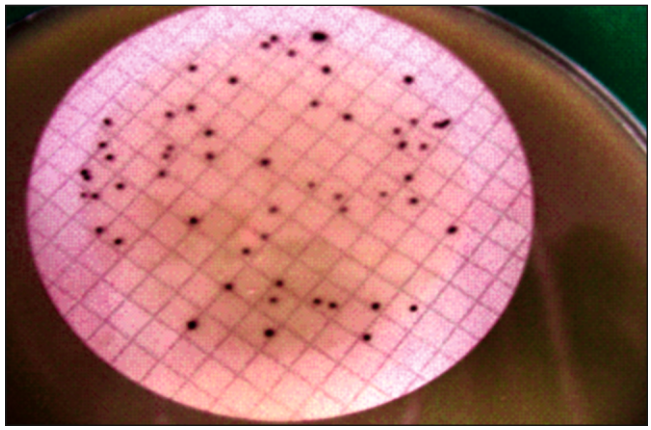

Fig. 6. The presence of presumptive *Clostridium perfringens* (black colonies)/plate with tryptose-sulfite-cicloserine (original)

Fig. 7. Acid phosphatase test (positive for *Clostridium perfringens*)(original)

The number of *Pseudomonas aeruginosa*/100 ml is a sum of number of confirmed colonies (blue-green colonies) (Fig. 8) and number of presumtive colonies that confirm by biochemical tests like: test of ammonia from acetamide (positive), oxidase test (positive) and fluorescence test (positive) on mediul King's B (positive) (Fig. 9).

In the present, in Romania, there isn't any separated standard for animal drinking, considering that water for animal drinking should have the same quality as water for human drinking (potable water). Therefore, the interpretation of these microbiological parameters is performed according with Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water (Tables 6, 7, 8), transposed form of EU Directive 98/83/EC on the quality of water intended for human consumption (Table 5).

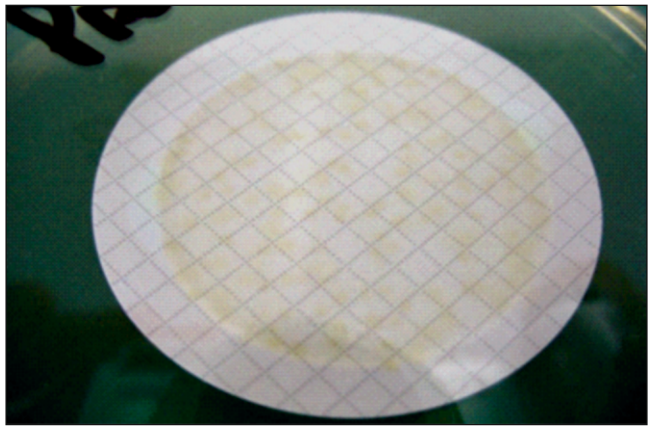

Fig. 8. The presence of confirmed *Pseudomonas aeruginosa* (blue-green colonies)/plate with Cetrimide agar (original)

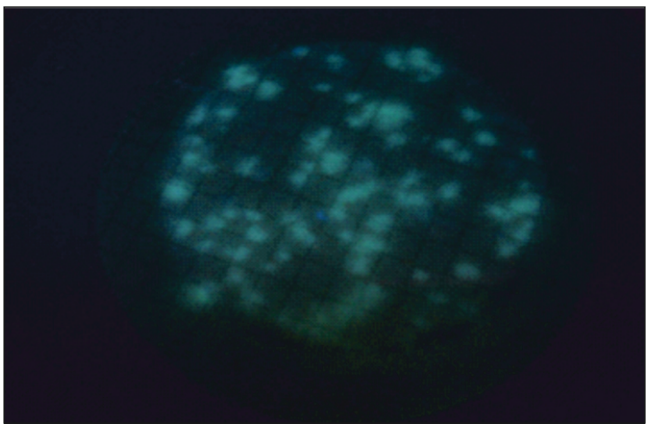

Fig. 9. The presence of confirmed *Pseudomonas aeruginosa* (fluorescent colonies)/plate with King's B agar (original)

The values of the indicator parameters were established for evaluation of drinking water quality by programme of monitoring performed by the regional authority of public health, in accordance with Norms for surveillance, sanitary inspection and monitoring of drinking water, developed by The Health Ministry.

In this normative act, there is no explication for terms "No abnormal modification", mentioned for parameters as: colony count 22°C and colony count 37°C (Table 8).

The establishing of drinking water quality is performed by correlations between the results of microbiological and biochemical analysis of water.

Table 5

EU Directive 98/83/EC on the quality of water intended for human consumption Annex 1 – Part A – Microbiological parameters

Parameter	**Accepted value**
Escherichia coli	0 ufc/100 ml
Enterococci	0 ufc/100 ml
The followings apply to water for sale in bottles/containers:	
Parameter	**Accepted value**
Escherichia coli	0 ufc/250 ml
Enterococci	0 ufc/250 ml
Pseudomonas aeruginosa	0 ufc/250 ml
Colony count 22°C	100 ufc/ml
Colony count 37°C	20 ufc/ml

Table 6

Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water - Annex 1 - Quality parameters of drinking water – Table 1A - Microbiological parameters

Parameter	**Accepted value**
Escherichia coli	0 ufc/100 ml
Enterococci	0 ufc/100 ml

Table 7

Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water - Annex 1 - Quality parameters of drinking water – Table 1B - Microbiological parameters for water offered for sale in bottles or containers

Parameter	**Accepted value**
Escherichia coli	0 ufc/250 ml
Enterococci	0 ufc/250 ml
Pseudomonas aeruginosa	0 ufc/250 ml
Colony count 22°C	100 ufc/ml
Colony count 37°C	20 ufc/ml

Table 8

Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water - Annex 1 - Quality parameters of drinking water – Table 3 - The indicator parameters

Parameter	**Maximum accepted concentration**
Coliform bacteria	0 ufc/100 ml
Clostridium perfringens	0 ufc/100 ml
Colony count 22°C	No abnormal modification
Colony count 37°C	No abnormal modification

CONCLUSIONS

1. The main microbiological parameters of drinking water are: *Escherichia coli* and enterococci, both indicating the recent faecal pollution.

2. The interpretation of analysis results of water intended for animal consumption is performed in accordance with Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water, because, in Romania, there is no a separated standard for water intended for animal consumption.

3. Law 311/2004 for modification and completion of Law 458/2002 concerning the quality of potable water isn't sufficiently clear for parameters: colony count 22°C and colony count 37°C for interpretation of obtained values; therefore it is necessary to improve this normative act.

4. EU Directive 2015/1787 for modification of EU Directive 98/83/EC on the quality of water intended for human consumption offers an update of methods for analysis of microbiological parameters: colony count 22°C and 37°C, coliform bacteria and *Escherichia coli,* intestinal enterococci, *Pseudomonas aeruginosa* and *Clostridium perfringens.*

5. The monitoring of water quality should be a major objective of The National Sanitary Veterinary and Food Safety Authority, in order to maintain the state of health and the level of animal production, to prevent outbreaking of transmissible diseases between animals and from animal to human, to protect the environment and, not at least, to protect human health.

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