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Application of redox potential measurement based technique for determination of total count in bottled mineral water

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1. INTRODUCTION

Food quality which includes water for consumption can be considered as a complex characteristic of food that determines its value or acceptability to consumers. The food must be, free of pathogens, food poisoning bacteria, their toxins and residues. Furthermore, food quality attributes include nutritional value, and organoleptic properties such as appearance, colour texture, taste and functional properties. In all countries the food industry bears the responsibility of meeting food quality and safety regulatory requirements. Contaminated food has severe outcomes such as food borne diseases as well as severe economic consequences.
Several documented diseases originating from water sources has occurred throughout Europe e.g. Campylobacter in Wales 2000 (Bartram 2003). This is why analyzing water for the presence of water borne pathogens has to be a standard practice in the mineral water industry.

The conventional bacterial testing methods, used for decades have relied almost exclusively on specific microbiological media to isolate and enumerate viable bacteria. This technique is complex and often requires specific media types for each targeted bacteria. It is also time consuming. Therefore at present there is a great demand for a fast, economic and precise microbiological method.

The purpose of this present study is the demonstration of the advantageous applicability of the redox potential based new method for detection of microbes present in bottled mineral water.

2. Survey of literature

2.1. Food Legislation

In general, the legislation in the European Union has four different forms. Regulations which are mandatory and legally binding those on the European community members. No alteration of national law is required for their implantation. Directives are these that are mandatory on the European community members. These will require several new laws for their implantation. Decision, apply specific rules for certain companies and individuals. Finally recommendations and opinions are not obligatory, but represent only an advice on good practices (European Commission, 2011).

The 852/2004 EU Regulation contains general hygiene requirements for all food businesses and covers a wide range of topics, including the general obligations of businesses in regard to food hygiene, the requirements for hazard analysis critical control point- (HACCP) based food safety management procedures, hygiene requirements for premises and equipment, staff training and personal hygiene, heat processes and packaging. It regulates also the primary production, thereby the whole food-chain is involved („field to table"). It declares that it is the producer/distributor who is responsible for food safety. Regulation (EC) No 178/2002 lays down general food safety requirements, according to which food must not be placed on the market if it is unsafe. Food Business operators have an obligation to withdraw unsafe food from the market. Regulation (EC) No 2073/2005 lays down the microbiological criteria for certain micro-organisms and the implementing rules to be complied with by the food business operators when implementing the general and specific hygiene measures referred to in the regulation (EC) No852/2004. Regulation (EC) No 852/2004 requires the business operator to keep and retain records and on request to make relevant information in these records available to the competent authority and receiving food business operator.

Mineral water is water that emerges from under the ground and then flows over rocks before it is collected. Unlike spring water, natural mineral water cannot be treated except to remove grit and dirt. Different brands of spring and mineral water contain differing amounts of minerals, depending on where they are sourced (Council Directive, 2009).

The Directive 2009/54/EC defines the provisions applicable to the marketing and exploitation of natural mineral waters.

Commission Directive 2003/40/EC of 16 May 2003 established the list, concentration limits and labeling requirements for the constituents of natural mineral waters and the conditions for using ozone-enriched air for the treatment of natural mineral waters and spring waters. Natural mineral waters are subject to an authorisation procedure carried out by the competent authorities of the EU member states or by European Economic Area (EEA) countries.

2.2. Traditional Microbial Methods

2.2.1 Pour Plate

Conventional testing methods of microbes make use of growth media and cultivation to enumerate and isolate microorganisms. Pour plate, is a method of melted agar inoculation followed by Petri dish incubation. It is used mainly for the enumeration of microorganisms, it often results in well separated discrete colonies which are available for the isolation of pure cultures with repeated plating or streaking. Quantitative microbiology is concerned with determining the concentration of microbial cells i.e. the number of living cells per ml or per gram of the sample. Enumeration is often made by the direct counting of colonies that have developed on the plates. It is assumed that each colony arises from a single cell that was originally inoculated in the medium however that is not always the case. Even after thorough homogenization, chains or clumps of cells may remain attached, and produce a single colony.
Therefore instead of cell counts, the term colony forming units (CFUs) per g or per ml is commonly used instead in food microbiology (Quinn et al, 2011).

2.2.2. Biochemical methods

Catalase, is an enzyme that is produced by many aerobes and facultative anaerobes, it causes the breakdown of hydrogen peroxide to oxygen and water. A positive oxidase test indicates the presence of cytochrome oxidase C in the bacterial cell. Reactions in oxidation-fermentation medium enables us to identify the atmospheric requirements of certain pathogens.
Biochemical tests relate to the catabolic activities of bacteria and an indicator system is usually employed to demonstrate the utilization of a particular substrate. Because the range of sugars utilized by individual bacterial species is usually limited, the catabolism of different sugars is frequently used for identification. (Quinn et al. 2011).

2.2.3. Immunological techniques

Serotyping is based on the immunological identification of surface antigens on pathogens such as *Escherichia coli* and other members of the Enterobacteriaceae as well as *Listeria monocytogenes*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. Immunological techniques such as fluorescent antibody staining is used for identifying bacterial pathogens. Antigen capture and direct enzyme linked immunosorbent assays have been developed for some bacterial pathogens and these require the immobilization of specific antibody on a solid phase. The bacterial agent, if present in the diagnostic specimen, is bound by the specific antibody and can be demonstrated by an enzyme-labeled antibody. Techniques using immune reactions can also be combined with other methods for improving detection of pathogens. Immunomagnetic separation, in which magnetic particles coated with antibodies to the particular pathogen bind the organism, combines physical and immunological methods. Immunomagnetic separation is usually followed by either cultural identification or molecular characterization of the organism. (Quinn et al. 2011).

2.2.4 Serology

Many potentially pathogenic bacteria are present as part of the normal flora of a host, these pathogens are also common in the environment. As animals are frequently exposed to these bacteria they may produce antibodies to the organisms. Antibodies demonstrable in a serum sample are, therefore, evidence that an animal was exposed to an infectious agent but they do not necessarily confirm an aetiological role for that agent. Despite these limitations, serological tests are used extensively for confirming infection with a particular pathogen in susceptible animals**.** (Quinn et al. 2011).

2.3. Rapid Microbial methods

As there are many alternative "rapid" methods in different formats presently available on the market, it can be very challenging for a food business operator or for a control authority to select the most appropriate method which fits its purpose (Jasson et al. 2010). Some of the more popular methods are listed below.

2.3.1 Molecular techniques

Certain molecular techniques can be used for the detection and enumeration of pathogenic bacteria. Molecular techniques assist in determining the virulence of an isolate by identifying genes associated with pathogenic properties. The main molecular biological techniques for the detection of pathogens are nucleic acid hybridization and the polymerase chain reaction (PCR). In nucleic acid hybridization, synthetic nucleic acid probes, specific for a particular pathogen, are applied either to prepared clinical specimens or to genetic material that has been extracted from the pathogen. Probes can be designed to detect DNA or RNA.

However, the usefulness of RNA probes is limited by the lability of the RNA molecule.

Diagnostic tests based on the detection of RNA can be particularly useful in specific areas such as food microbiology because they allow the discrimination of viable from dead microorganisms. Probes can be designed to detect all members of a particular genus or to detect strains within a species. Assays based on the direct detection of DNA or RNA are relatively insensitive because they usually require large numbers of bacteria (10^4 to 10^5) in the given specimen. For specimens containing a limited number of bacteria, amplification of the nucleic acid of the target organisms by PCR can be used. After amplification of a specific fragment of DNA, using either a DNA or RNA template, the PCR product can then be identified by its electrophoretic pattern using a appropriate size-marker molecules. Restriction endonuclease analysis and gene probes are two methods that are currently employed for epidemiological investigations. The selected technique must be convenient to use and must have the ability to discriminate between closely related strains by detecting genetic differences of epidemiological significance. Restriction endonucleases can be used to cleave chromosomal or plasmid DNA to generate fragments that can then be separated by gel electrophoresis. Restriction enzymes which cleave DNA in only a few places produce large fragments which can be separated using pulsed-field gel electrophoresis, a method that is frequently used in epidemiological studies (Quinn et al. 2011).

2.3.2 Impedimetric methods

Until now, impedance-measurement has been the preferred method for rapid quantitative and qualitative measurements of bacteria, yeasts and moulds and also for monitoring real time activity. The method is based on detection of a change of impedance of a special broth, which develops when the microorganisms proliferate within the broth.

Normal metabolic pathways of the test organisms will convert weakly charged substrates of the medium into highly charged end products, leading to an increased conductivity of the test medium. This means that the medium used in the impedimetric method is extremely important: as it must be supportive as well as selective for the test organism's growth, and in addition must be optimized for electrical signals. (Reichart, 2010)

2.3.3. MicroTester

The possibility of the redox potential measurement has been known since the end of the 19th century, however it had not been used until more recently for the enumeration of the viable microorganisms. The new measuring system (MICROTESTER) developed by the workers of Szent István University, Faculty of Veterinary Science, Department of Food Hygiene and Corvinus University, Faculty of Food Science, Department of Physics and Automation is based on redox-potential measurement. The evaluation of the changes of the measured values provides the possibility for a more detailed determination of the viable cell count in comparison to impedimetric methods. This method utilizes the typical oxidation-reduction reactions in biological systems, performed by microorganisms. The energy source of the microbial growth is the biological oxidation which leads to a reduction in the environment. This is due to the oxygen depletion and the production of reducing compounds in the nutrient medium. The redox potential is one of the more complex indicators of the physiological state of microbial cultures and its measurement is of great benefit for qualitative and quantitative determination of the microbial contamination in a given sample (Szakmár, 2009). Therefore the detection of the redox-potential changes provides a unique opportunity to determine the nature of the microbe (aerobe, anaerobe, facultative anaerobe, aero-tolerant anaerobe) (Reichart, 2010).

MICROTESTER can be used favorably for evaluating the classical membrane-filtration and surface swabbing methods.

During inspection of the microbial count of natural water sources, drinking water, mineral water and other beverages usually consecutive members of the dilution series are membrane-filtered and followed by placing the filters on agar surface, the samples are then incubated. The reason for using different dilutions in the classical method is that the samples can only be reliably evaluated in a specific interval (between 30-300 cells). Because the MICROTESTER – with determining the calibration curves – is able to carry out measurements and reliably determines the cell concentration in a wide interval (between 1-10^7 cells), there is no need for dilution series; it is sufficient to make only a single membrane filtration from a sample (Reichart, 2010).

2.4. Mineral Water

Many consumers use mineral water as an alternative to tap water and find it safer and more delectable (Osman, 2009).

Naturally sourced waters are the most popular choice across the European Union: natural mineral water and spring waters together represented 97 % of the market volume in 2009.

Per capita consumption of bottled water in the European Union varies enormously from one country to another with the average consumption at 105 litres per year.

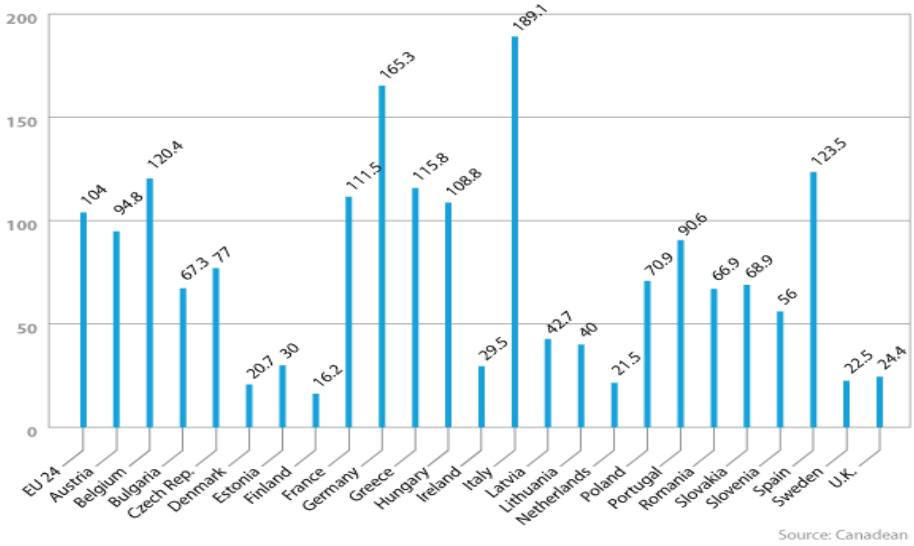

Figure 1. - EU and individual country per capita consumption in 2009 (litres)
(European Federation of Bottled Waters, 2010) .

As there is such a significant consumption of Mineral water throughout Europe and the world there has been many various regulations enforced to ensure the safety of the consumers, as many various pathogenic microbes can contaminate and cause various illnesses and even death.

2.5. Common waterborne pathogens

Waterborne disease is a global burden which is estimated to cause more than 3 million deaths and countless cases of sickness every year. Although the extent of the problem is hard to determine and numbers tend to be a gross underestimation due to the lack of a surveillance system, the World Health Organization (WHO) estimated in 1996 that water-related disease caused the death of one child every 8 seconds (WHO, 1996). Most of these cases occur in the Developing countries however there are frequent outbreaks occurring throughout Europe to this day.

2.5.1 Recognized waterborne pathogens

Water borne diseases are typically caused by enteric pathogens of which most are transmitted via the oral fecal route. Some of these pathogens are of animal origin. Water may also be important in the transmission of opportunistic pathogens from wounds, lesions or ulcers or may be present in water as it is a natural habitat (Grabow,1996).

The human pathogens that can be transmitted orally via drinking-water are listed in figure two below (compiled from data provided by the Communicable Disease Surveillance Centre), together with a summary of their health significance and main properties. (Bartram, 2003).

Pathogen	Health significance	Persistance in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Bacteria					
Burkholderia pseudomallei	High	May multiply	Low	Low	No
Campylobacter jejuni	High	Moderate	Low	Moderate	Yes
E.coli pathogenic	High	Moderate	Low	Low	Yes
E.coli Enterohaemarrohagic	High	Moderate	Low	High	Yes
Legionella sp	High	May multiply	Low	Moderate	No
Non tuberculos mycobacteria	Low	May multiply	High	Low	No
Pseudomonas aerguginosa	Moderate	May multiply	Moderate	Low	No
Salmonella typhi	High	Moderate	Low	Low	No
Other Salmonella	High	May multiply	Low	Low	Yes
Shigella spp	High	Short	Low	High	No
Vibrio Cholerae	High	Short to long	Low	Low	No
Yersinia enterocoliticia	High	Long	Low	Low	Yes
Viruses					
Adenoviruses	High	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Astroviruses	High	Long	Moderate	High	No
Hepatitis A viruses	High	Long	Moderate	High	No
Hepatitis E viruses	High	Long	Moderate	High	Potentially
Noroviruses	High	Long	Moderate	High	Potentially
Sapoviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Protozoa					
Acanthamoeba spp	High	May Multiply	Low	High	No
Cryptosporidium parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naegleria fowleri	High	May Multiply	Low	Moderate	No
Toxoplasma gondii	High	Long	High	High	Yes

Figure 2. (Bartram, 2003)

2.5.1.1 *Campylobacter*

Campylobacter are the most common cause of human bacterial gastroenteritis in Great Britain, with *Campylobacter jejuni* being the predominantly isolated species. They are widespread in the environment and occur very frequently in the intestinal tracts of animals, including birds.
Ninety-five per cent of ready-prepared chickens are contaminated with Campylobacter, and poultry meat is thought to be an important leading source of infection. Wild birds also have a high intestinal colonization rate. Campylobacter can easily be isolated from surface waters. (Bartram, 2003).

2.5.1.2 *Escherichia coli*

Most *E. coli* are not pathogenic and are part of the normal human bowel flora.
Some types possess virulence factors and cause gastroenteritis in humans by several different mechanisms. Seven such groups have been defined, of which three may be waterborne (Food Standards Agency 2000):

- Enteropathogenic E. coli have been associated with outbreaks in children in nurseries and hospital wards. These strains belong to particular "O" serotypes.
- Enterotoxigenic E. coli are a common cause of diarrhoea in travelers. They are identified by the production of a heat-stable toxin and a heat labile toxin.
- Verocytotoxigenic E. coli (VTEC) cause serious diarrhoeal disease, with bloody diarrhoea and painful abdominal cramps. In 10–15% of cases, haemolytic uraemic syndrome develops as a complication, which can result in kidney failure or even death. The most frequent serotype isolated is O157, but other serotypes, such as O139, have been reported.

The organism is common in farm animals, it behaves as a commensal organism and does not cause any recognized disease. The infectious dose for VTEC is very low, about 10–100 organisms, which explains their potential to cause waterborne outbreaks when animal faeces-contaminated material gains access to water supplies past treatment or where treatment is inadequate. Indicator organism tests will indicate the potential for the presence and survival of pathogenic E. *coli*. Fortunately, these organisms are highly susceptible to water disinfection techniques. (Bartram, 2003).

2.5.1.3 *Salmonella*

The salmonellas cause two distinct types of disease. One group of two species, *Salmonella typhi* and *Salmonella paratyphi*, is the cause of the enteric fevers, typhoid and paratyphoid. The other group, consisting of more than 2000 serotypes of what is now considered to be one species, *Salmonella enterica*, causes gastroenteritis. These serovars were previously considered to be separate species and were named after the city or animal from which the organism was initially isolated. Transmission of salmonellas is by the fecal oral route and often involves food and sometimes even water. The enteric fever salmonellas are associated only with humans and human disease and remain important causes of waterborne disease throughout the world, but nowadays very rarely in developed countries. The gastroenteritis salmonellas are widespread in animals and are often found in poultry, eggs and meat products. Food is the predominant vehicle of infection, but transmission via water does sometimes occur, even though the bacteria survive only for a relatively short period of time, a few hours or days in surface water. Normal water treatment processes are sufficient to remove the organism from drinking-water. The organisms are susceptible to chlorine disinfection. The infectious dose for humans for the enteric fever salmonellas is about 10^2–10^3 organisms, whereas the infectious dose for humans for the gastroenteritis salmonellas is about 10^6–10^8 organisms, mainly because of their susceptibility to gastric acid. The enteric fevers are systemic infections that present with high fever (40–41 °C), headache, malaise and rigors. Diarrhoea does not usually occur, and patients often experience constipation in early enteric fever. (Bartram, 2003).

2.5.1.4 *Shigella*

Species of Shigella are the causative organisms of dysentery and are almost entirely human pathogens; no other animal species play a role in maintenance or spread of infection in the community. Occasionally, higher primates become infected by transmission from human to animal. Shigellas are transmitted by the fecal–oral route and sometimes, because the infectious dose is low, around 10^2 organisms it is spread from person to person. Patients excrete large numbers of organisms, between 10^5 and 10^8per gram of feces (Bartram, 2003).

2.5.1.5 *Vibrio*

The Vibrio genus is composed of over 30 species, of which the most important is *V. cholerae*, which causes epidemic cholera, a predominantly waterborne infection. The species *V. cholerae* is subdivided into 140 O-serovars, of which the toxin-producing strains are O1 and O139. The epidemiological picture of cholera has changed and now has a wide distribution (Bartram, 2003).

2.5.2. Emerging pathogens

Although many of the established waterborne pathogens have been controlled by sanitation measures and water treatment processes, new diseases continue to be identified, and new discoveries present a better understanding of existing chronic diseases. Many of these discoveries raise questions about possible waterborne transmission. The bacteria that now need to be considered in this developing area are *Helicobacter pylori*, *Mycobacterium* species, *Burkholderia pseudomallei* and *Francisella tularensis*. In the past 40 years a number of significant pathogens that can be water-borne including rotavirus, norovirus, *V. cholerae 0139, Cryptosporidium,* have been emerging. Many more are classified as emerging due to detection of increased incidence of disease or detection in areas where they were not previously established. The emergence of infectious diseases, including those that are water-borne, is caused by many various factors such as population growth, migration, travel, new environments, climate change, improved methodology and drug resistance (Cunliffe, 2008).

2.5.2.1 *Helicobacter pylori*

Although spiral-shaped organisms have been observed in the stomachs of humans for many years, it was not until 1982 that a Campylobacter-like organism was isolated from patients with gastritis and a causative relationship between a new species, *Helicobacter pylori*, is a pathogen of global proportions and is generally accepted as the cause of most gastric and peptic ulcers. These diseases may lead to gastric adenocarcinoma. *H. pylori* occur worldwide in developing and developed countries. Where low degrees of hygiene and socioeconomic problems exist, infection rates may reach 100%. In developed countries, infection rates are approximately between 30 and 60%. Transmission from person to person at present is not fully understood, mainly because of the difficulty in culturing the organism and identifying it outside of the body.

Epidemiological studies show the cluster phenomenon of *H. pylori* infection in families.
H. pylori has been identified in feces, and it is assumed that transmission is therefore via oral–oral or faecal–oral route. The organism has not been isolated from the environment or from drinking water, and waterborne transmission remains a possibility that should be investigated.
The epidemiology, however, points to person-to-person transmission in early life. (Bartram, 2003).

2.5.2.2. *Mycobacterium*

The mycobacteria are a group of slow-growing organisms. The most important is *Mycobacterium tuberculosis*, the causative organism of tuberculosis, which takes about 4–6 weeks to grow in the diagnostic laboratory. *M. tuberculosis* is not a waterborne pathogen; there are, however, a number of Mycobacterium species that occur in the environment and can cause disease in humans. *Mycobacterium avium* and its related species cause an infection of cervical lymph nodes; it occurs in the environment and is most probably accompanied by ingestion or inhalation. *M. avium* can grow in water to which no additional nutrients have been added; although water treatment processes of coagulation and filtration appear to reduce the numbers, it is not affected by chlorine levels of 1 mg/ml. It is therefore not surprising that these organisms can regrow and colonize domestic water systems. Once ingested, *M. avium* can colonize the pharynx without causing any disease. The number of cases reported was very low, but patients with HIV/AIDS are very susceptible. *Mycobacterium paratuberculosis* causes Johne's disease in cattle. It is a chronic wasting disease with considerable economic consequences. The organism is extremely difficult to culture; when it does grow, it is very slow and dependent on an exogenous source of mycobactin, which is an iron chelating agent produced by all other mycobacteria. Transmission is by either direct or indirect contact with infected animals and occurs mainly through the faecal–oral route. Organisms are ingested in large numbers by young animals when they feed in troughs that have been contaminated by feces of shedding animals *M. paratuberculosis* has recently been suggested as a cause of Crohn's disease, a non-specific chronic transmural inflammatory disease of humans that affects the intestinal tract, commonly the ileum. The disease is chronic, debilitating and of a relapsing nature; the symptoms experienced include diarrhea with blood in the stools and abdominal pain. There have been many bacteria implicated over the years, but no definite etiological agent has been found (Bartram, 2003).

2.5.2.3. *Francisella tularensis*

Tularaemia is a zoonosis caused by a highly infective and virulent organism *Francisella tularensis*, which occurs throughout the northern hemisphere but has never been isolated within the United Kingdom. It occurs in a wide range of animal reservoir posts and can be isolated from the environment in water and mud. It is transmitted to humans who come in close contact with the animal reservoir, arthropods that feed on them or debris and dust associated with them. It can also be transmitted through the ingestion of contaminated water.

Human epidemics sometimes occur and are associated with epizootics in the animal populations, evidenced by die-offs. There are several presentations of tularaemia in humans, depending on the route of exposure. Ingestion usually results in oropharyngeal tularaemia, with fever, pharyngitis and cervical lymphadenitis. Other forms include ulcero-glandular, pleuropneumonic and typhoidal. Following the recent war in Kosovo, close to 1000 suspected cases of tularaemia were identified and 327 cases was confirmed serologically. The epidemiological investigation pointed to rodent-contaminated wells, and rodent carcasses found in some wells tested positive for *F. tularensis*. (Bartram, 2003).

2.6. Microbial Detection in mineral water

The microbiological analyses required to comply with the *Natural Mineral Water Regulations* are similar to those required under the water in containers regulations, except that sample volumes for most parameters are greater (250 ml). Colony count standards apply to water sampled within 12 hours. After 12 hours the colony count should be no more than that which results from the normal increase in the bacterial content of the water at source. Colony count (aerobic) Clostridia Coliforms Enterococci and *Escherichia coli* (Roberts et al. 2003).

On 15th July 1980 Council Directive 80/777/EEC on the approximation of the laws of the Member States relating to the exploitation and marketing of natural mineral waters was established. Since then new Directives has been enforced on the total colony count that is acceptable for human consumption.

According to directive 2009/54/EC of the European Parliament and of the council of 18 June 2009 on the exploitation and marketing of natural mineral waters. The revivable total colony count of a natural mineral water at source shall conform to its normal viable colony count and give satisfactory evidence of the protection of the source against all contamination.

After bottling, the total colony count at source may not exceed 100 per milliliter at 20 to 22 ºC in 72 hours on agar-agar or an agar-gelatine mixture and 20 per milliliter at 37 ºC in 24 hours on agar-agar.

The total colony count shall be measured within the 12 hours following bottling, the water being maintained at 4 ºC ± 1 ºC during this 12-hour period.

At source, these values should not normally exceed 20 per milliliter at 20 to 22 ºC in 72 hours and 5 per milliliter at 37 ºC in 24 hours respectively, on the understanding that they are to be considered as guide figures and not as maximum permitted concentrations.

2. At source and during its marketing, a natural mineral water shall be free from:

(a) Parasites and pathogenic micro-organisms;

(b) *Escherichia coli* and other coliforms and faecal streptococci in any 250 ml sample examined

(c) Sporulated sulphite-reducing anaerobes in any 50 ml sample examined;

(d) *Pseudomonas aeruginosa* in any 250 ml sample examined.

Summary of microbiological limits for spring waters at source and in package.

Note: Legislative limits are specified for the parameters which are subject to audit monitoring. Indicator values are specified for the parameters which are subject to check monitoring. Exceeded indicator values are used as a prompt to investigate further.

- TVC @ 20-22°C for 72hrs 20 cfu/ml* 100cfu/ml** Daily Article 5(1)of Directive 2009/54/EC
- TVC @ 37°C for 24hrs <5 cfu/ml* <20cfu/ml** Daily Article 5 (1)of Directive 2009/54/EC
- Coliforms @ 37°C 0 in 250mls 0 in 250mls Daily Article 5 (2b)of Directive 2009/54/EC
- *Escherichia coli* @ 44.5°C 0 in 250mls 0 in 250mls Daily Article 5 (2b) of Directive2009/54/EC
- Sporulated sulphite reducing anaerobes 0 in 50 mls 0 in 50mls Monthly Article 5 (2c)of Directive2009/54/EC
- Enterococci (faecal streptococci) 0 in 250mls 0 in 250mls Daily Article 5 (2b) of Directive2009/54/EC
- *Pseudomonas aeruginosa* 0 in 250mls 0 in 250mls Monthly Article 5 (2d) of Directive2009/54/EC
- Parasites and pathogenic microorganisms Absent Yearly Article 5 (2a) of Directive2009/54/EC

* Figures are legislative guide figures ** Samples must be measured within 12 hours of bottling and maintained at 4 ± 1°C during this 12 hour period.*** Sourced from IS 432 of 2010 (Food Safety Authority of Ireland, 2010)

3. OBJECTIVE OF THE STUDY

The Objective of this study is to determine the microbial count of two brands of bottled mineral water; both sparkling and still mineral water will be examined by the use of classical culture method and by the use of redox potential measurement method.

The appropriate time required to produce results using the redox potential technique will be compared to the traditional pour plate method to determine which is the most efficient.

4. MATERIALS AND METHODS

4.1 Media

PC agar (Plate-count agar)

Tryptone	5,0 g
D-glucose	1,0 g
Yeast extract	2,5 g
Agar-agar	14,0 g
Distilled water	1000 ml

Pepton Water

gelatine pepton	1,0 g
NaCl	8,5 g
Distilled water	1000 ml

1/2 TSB (Tryptic Soy Broth)

Casein pepton	17,0
Soy pepton	3,0
d-glucose	2,5
NaCl	5,0
K_2HPO_4	2,5
Distilled water	1000 ml

4.2 Examination methods

4.2.1 Determination of viable count the plate method

According to the prescription of standard ISO 6222:1999 Water quality. Enumeration of culturable micro-organisms. The diluting medium was peptone-containing physiological salt.
The applied culture media was Plate Count (MERCK) agar. (MSZ EN ISO 6222:2000).
The pour plate technique can be used to determine the number of microbes/ml or microbes/gram in a specimen. It has the advantage of not requiring previously prepared plates, and is often used to assay bacterial contamination of foodstuffs.

4.2.2 Preparation of sample

- Placing of 10ml of sample from the bottled mineral water into a labeled Petri dish. Each sample had a separate Petri dish, four in total (still number one, still number two, sparkling mineral water number one, sparkling mineral water number two).
- 15ml of Melted Agar which has been cooled to 45c was added and was swirled gently so as the sample and agar would mix well.
- The Petri dishes was then left to cool until they solidified on a flat table top.
- The samples was then inverted and incubated to allow for colonies to develop.

4.3 Instrumental method based on redox potential measurements

The principle of the measurement is that due to the energy producing biological oxidative processes of the multiplicating bacteria, the redox potential of the medium is reduced at a certain microbe concentration which is easily detected. The detection time (TTD) is the moment when the absolute value of the rate of redox potential change in the measuring-cell overcomes a value which is significantly different from the random changes (e.g. |dE/dt| 0.5 mV/min). This value is the detection criterion. There is a close linear correlation between TTD and the logarithm (log N) of the initial viable count that is represented by the calibration curve.

The microbe count can be calculated from the TTD value by the calibration curve. This method provides a good estimation of the microbial count and additionally, owing to the characteristic pattern of the redox curves, it allows for the possibility for identifying the composition of the microflora. (Reichart, et al. 2007).

The determinations were carried out by using a 16-channel measuring instrument. The MicroTester system was developed and patented by the researchers of the Department of Food Hygiene (Szent István University, Faculty of Veterinary Science) and of the Department of Physics and Automatization (Corvinus University, Faculty of Food Science). The parts of the MicroTester system comprises of: a water bath, thermostat (that is accurate to: ± 0.2 °C), test cells, which in this case used were 30 ml test tubes that was equipped by Schott BlueLine 31 RX redox-electrodes, PC drive, (Windows XP, special data collection software and evaluating unit aswell as a monitor.

4.4 Samples

We examined 1,5 litre (PET bottle) still and sparkling mineral water samples, of which was bought commercially. The samples were bought at separate occasions, and were also bought at various locations to ensure the water samples were from separate batches.
Two brands was selected, one more expensive and a cheaper mineral water in hopes that the two samples would be of different microbial quality.
In order to identify each sample the cheaper mineral water brand was labeled with number 1 and the more expensive brand was labeled with number 2.

4.5 Determination of the calibration curve

Decimal dilution series was prepared from the examined sample. The “0” dilution degree was 1000 ml water. Time to detection (TTD) belonging to the dilution degrees was determined.
The total count of microbes present in the water samples was determined by plate pouring method or in the case of low cell number by membrane filtering. From the count of microbes the lgN values belonging to each dilution degree was calculated. From the lgN-TTD pairs the calibration curve could be constructed.

4.6 Procedure

The electrodes was disinfected in 3% hydrogen peroxide, they were then rinsed in 70% ethanol and immersed aseptically into the test tubes containing the mineral water samples.

The test tubes were then placed into the water bath and were connected to the data collector.

The software was initialized and measured. The Time to Detection was automatically determined.

Upon completion of the measurements the electrodes was disinfected in Na hypochlorite 1:10 for 30 minutes and rinsed in water.

5. RESULT AND DISCUSSION

5.1. Results of the still mineral water

Calibration curves of the two kinds of still mineral water were determined at three different occasions. The basic results of the measurement are summarized in Table 1.

Table 1. Result of the measurements of still mineral water samples

Still water sample 1

Date	02.22.			03.01.			03.08.		
Filtered volume (ml)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)
1000	4.13	10.5		3.99	11.2		4.31	10.83	
100	3.13	13.17		2.99	14		3.31	12.67	
10	2.13	15.17	135	1.99	16.17	97	2.31	15.17	202
1	1.13	17.5		0.99	18.17		1.31	17	
0.1	0.13	21.17					0.31	20.17	
0.01									

Still water sample 2

Date	02.22.			03.01.			03.08.		
Filtered volume (ml)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)
1000	2.75	15		2.52	15.5		2.18	16.17	
100	1.75	16.33	56	1.52	17	33	1.18	18	15
10	0.75	18.67		0.52	19.17		0.18	20.5	
1									

Correlation between the logN – TTD measured results allowed for the determination of the calibration curves.

The calibration curves are shown in Figure 3. and 4.

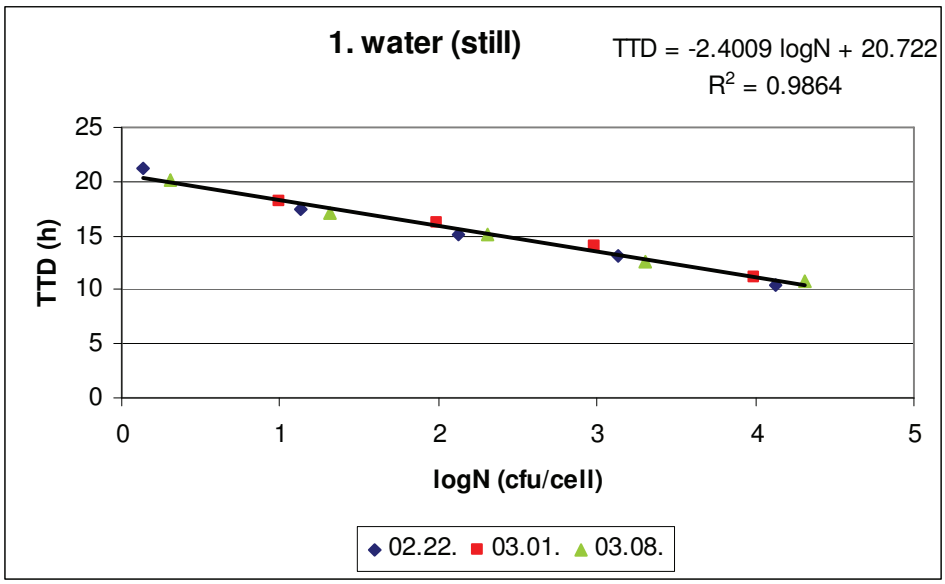

Figure. 3. Calibration curve of still mineral water labeled number one

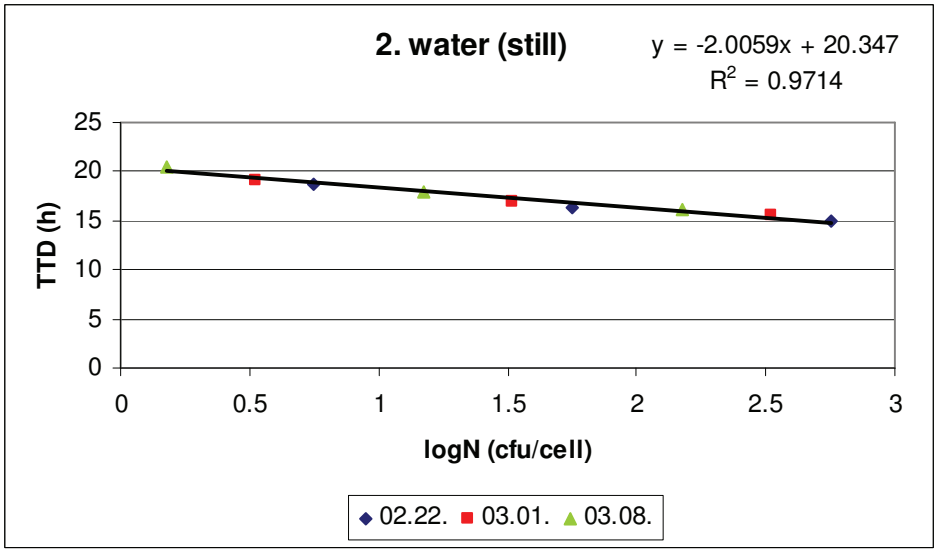

Figure 4. Calibration curve of still mineral water labeled number two

The common calibration curve is shown in Figure.5.

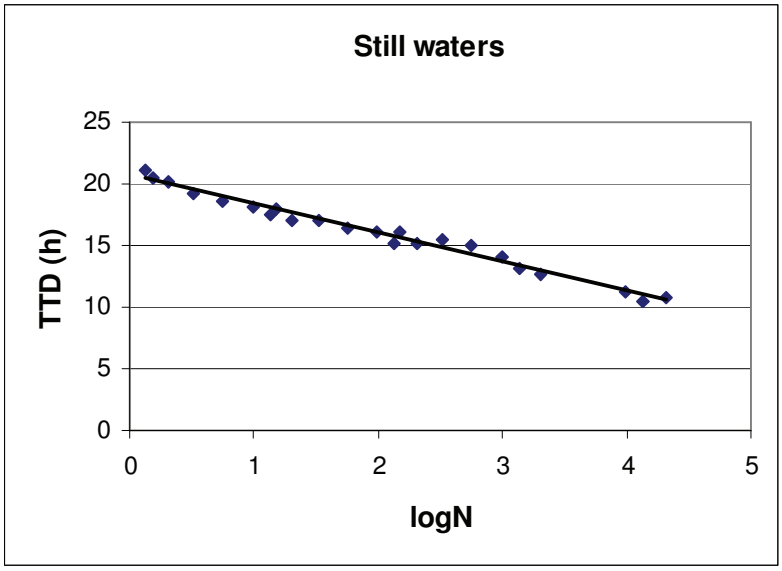

Figure 5. Common calibration curve of still mineral water samples

We can evaluate the data by analysis of the covariance that the calibration curve of the two types of water sample "sparkling and still" can be described with a common equation.

Table 3. Data of the common calibration curve of still mineral waters

Regression coefficient calculated from all datapairs (slope) :	b =	-2.3550
Intercept calculated from all datapairs	a =	20.7134
Standard error (random error,) :	Syx =	0.4340
coefficient of determination :	R^2=	0.9803
Correlation coefficient :	R =	-0.9901

During the redox potential measurement we measured the TTD values, from these measurements we were able to calculate the logarithm of the microbial count.

TTD = a - b·logN

logN = A - B·TTD $A=\frac{a}{b}$; $B=\frac{1}{b}$

The calibration curve: $logN = 8{,}7924 - 0{,}3842 \cdot TTD$

5.2. Results of the sparkling mineral water

We determined the calibration curves of the two kinds of sparkling mineral waters.

The basic data of the measurement are summarized in Table 4.

Table 4. Data of measurement of sparkling mineral water

Water Sample 1 (sparkling)

Date	02.22.			03.01.			03.08.		
Filtered volume (ml)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)
1000	3.28	14.5		3.38	14.33		3.59	14	
100	2.28	16.83		2.38	16.5		2.59	16	
10	1.28	19.5	19	1.38	19.17	24	1.59	18.5	39
1	0.28	22.17		0.38	22		0.59	20.87	
0.1									

Water Sample 2 (sparkling)

Date	02.22.			03.01.			03.08.		
Filtered volume (ml)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)	logN (cfu/cell)	TTD (h)	N (cfu)
1000	2.04	17.17		2.18	16.83		1.95	18	
100	1.04	20.33	11	1.18	20	15	0.95	21.17	9
10	0.04	22.5		0.18	22		0.10	23.17	
1									

From the data of measurement logN – TTD connection was determined,

The calibration curves are shown in Figures. 6 and 7.

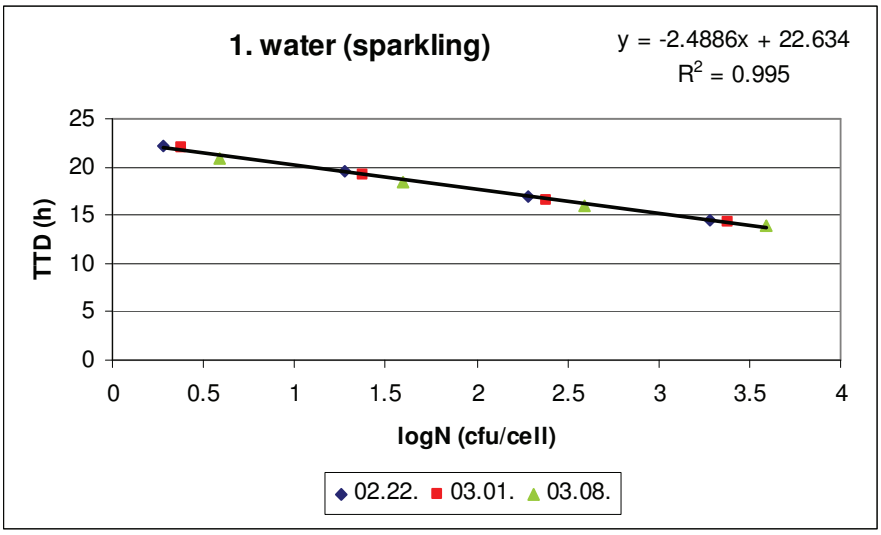

Figure 6. Calibration curve of sample No 1. sparkling mineral water

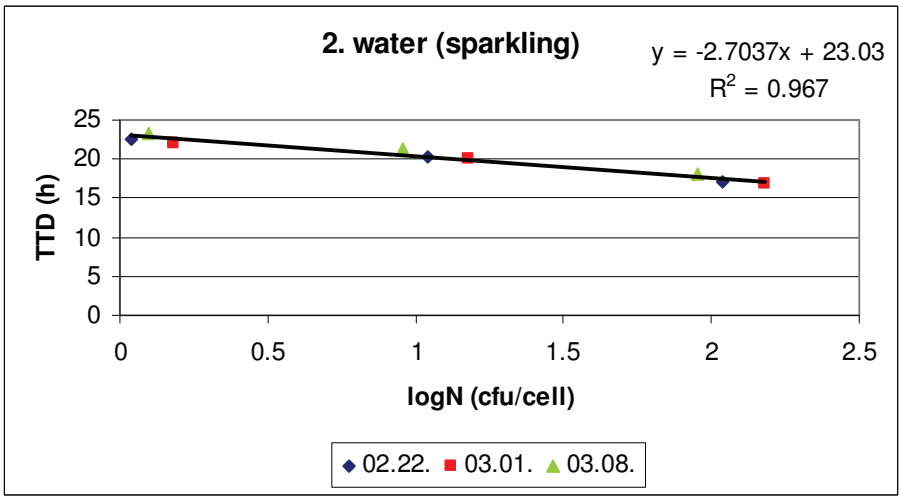

Figure 7. Calibration curve of sample no 2. sparkling mineral water

The common calibration curve is shown in Figure 8.

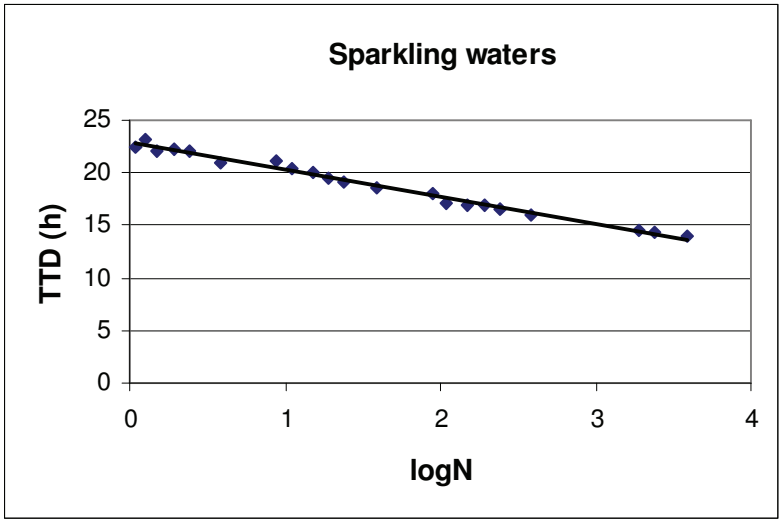

Figure 8. Common calibration curve of sparkling mineral waters

The data of the common calibration curve are summarized in Table 5.

Table 5. Data of the common calibration curve of sparkling mineral water

Regression coefficient calculated from all data pairs (slope):	b =	-2.5682
Intercept calculated from all data pairs:	a =	22.8289
Standard error (random error):	Syx =	0.3377
coefficient of determination:	R^2=	0.9868
Correlation coefficient:	R =	-0.9934

During the redox potential measurement we measure the TTD values, from these we calculated the logarithm of the microbe's count.

TTD = a - b·logN

logN = A - B·TTD A=$\frac{a}{b}$; B= $\frac{1}{b}$

The calibration curve:

logN = 8,6608 – 0,4162·TTD

5.3. Accuracy of the MicroTester results

Using the calibration curve we determined the microbial count of the two kinds of still and sparkling mineral water that was bought at three different locations. The measurement: 100 ml water membrane filtering, after filtering the paper membrane was placed into a cell containing 15ml broth. The equation of the calibration curve was loaded into the equipment and the measurement was then started.

The equipment determines the TTD (hour) and based on the calibration curve calculates the count of microbes of the sample.

To check the accuracy of the measurement with the equipment, we determined the microbial count by plate pouring method also.

5.3.1. Experimental results of the still mineral water samples

Table 6. Results of still mineral water samples

Sample 1. water, still						
	MicroTester filtration: 100 ml				Classical method Plate count: 1 ml	
Date	logN			N		N
	TTD (h)	(cfu/cell)	N (cfu/cell)	(cfu/ml)		(cfu/ml)
2012.03.12	13.67	3.54	3470	35	-	45
	13.5	3.61	4034	40	-	35
	13.5	3.61	4034	40	-	39
2012.03.19	12.5	3.99	9770	98	-	133
	12.33	4.06	11356	114	-	129
	12.5	3.99	9770	98	-	130
2012.03.22	13.83	3.48	3012	30	-	38
	13.5	3.61	4034	40	-	32
	13.17	3.73	5401	54	-	34

Sample 2. water, still						
	MicroTester filtration: 100 ml				Classical method filtration: 10 ml	
Date	TTD (h)	logN (cfu/cell)	N (cfu/cell)	N (cfu/ml)	N (cfu/10ml)	N (cfu/ml)
2012.03.12	17	2.26	182	1.8	21	2.1
	17.17	2.20	157	1.6	12	1.2
	17	2.26	182	1.8	23	2.3
2012.03.19	16.21	2.56	367	3.7	30	3.0
	16.33	2.52	330	3.3	28	2.8
	16.17	2.58	380	3.8	32	3.2
2012.03.22	18.5	1.68	48	0.5	8	0.8
	18	1.88	75	0.8	5	0.5
	18.17	1.81	65	0.6	4	0.4

Results determined by MicroTester and classical method were compared. (shown in Figures. 7. and 8.) SD (Standard Deviation) values of the measurement with the equipment are from the analysis of co-variance.

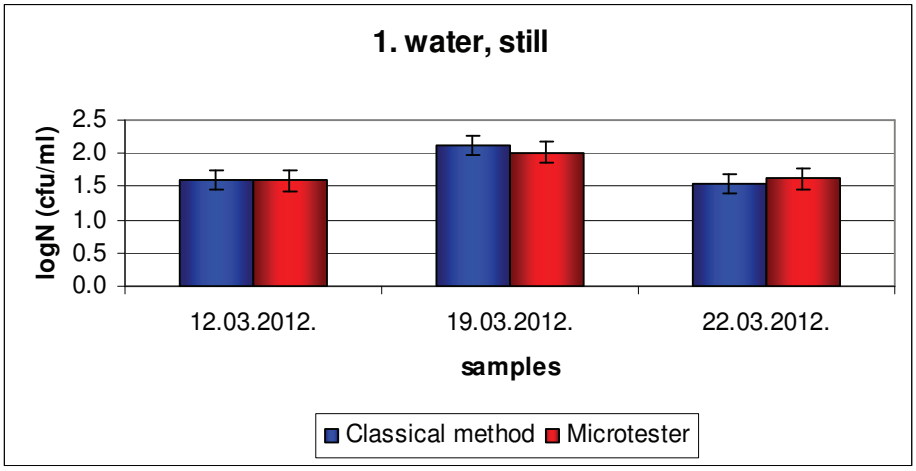

Figure 9. cell concentration of No 1 water samples (lgN ± SD)

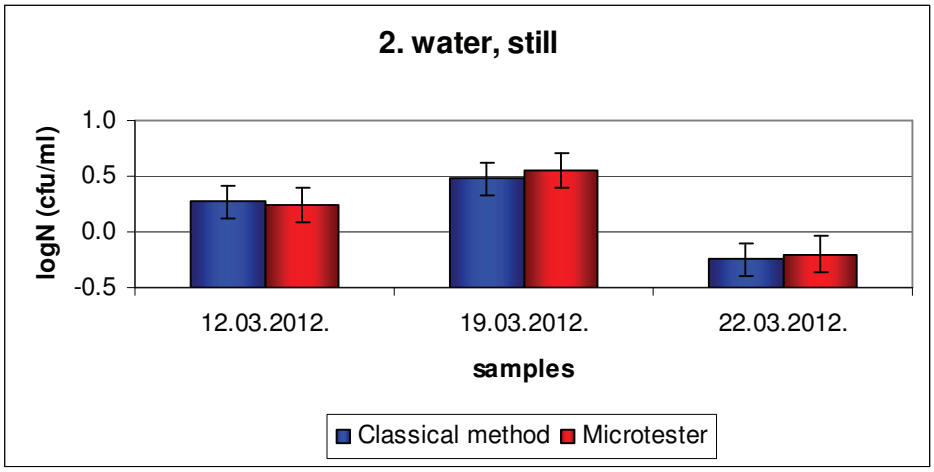

Figure 10 microbial count of no 2 water samples (lgN ± SD)

It can be concluded that no significant difference between the classical and redox method was observed.

5.3.2. Experimental results of the sparkling mineral water samples

Results are summarized in Table 7.

Table 7. Results of the sparkling mineral water samples

1. water, sparkling						
	Microtester filtration: 100 ml				Classical method filtration: 10 ml	
Date	logN			N	N	N
	TTD (h)	(cfu/cell)	N (cfu/cell)	(cfu/ml)	(cfu/10ml)	(cfu/ml)
2012.03.12	14.17	2.76	580	5.8	53	5.3
	14	2.83	682	6.8	58	5.8
	14.17	2.76	580	5.8	55	5.5
2012.03.19	15.5	2.21	162	1.6	18	1.8
	15.17	2.35	222	2.2	25	2.5
	15.33	2.28	191	1.9	19	1.9
2012.03.22	15	2.42	262	2.6	28	2.8
	15.17	2.35	222	2.2	25	2.5
	15	2.42	262	2.6	26	2.6

2. water, sparkling						
	MicroTester filtration: 100 ml				Classical method filtration: 10 ml	
Date	logN			N	N	N
	TTD (h)	(cfu/cell)	N (cfu/cell)	(cfu/ml)	(cfu/10ml)	(cfu/ml)
2012.03.12	20.17	0.27	1.8	0.02	0	-
	20	0.34	2.2	0.02	0	-
	20.33	0.20	1.6	0.02	0	-
2012.03.19	20.5	0.13	1.3	0.01	0	-
	20	0.34	2.2	0.02	0	-
	20.17	0.27	1.8	0.02	0	-
2012.03.22	20.5	0.13	1.3	0.01	0	-
	20.17	0.27	1.8	0.02	0	-
	20.5	0.13	1.3	0.01	0	-

In the case of the 1st water sample the results of the classical method and the results of the MicroTester measurement were compared. It is shown in Figure 9. The SD values of the measurement with the equipment are from the analysis of the covariance.

Microbes could not be detected in the 2nd water sample with the 10 ml membrane filtering, therefore a comparison could not be done.

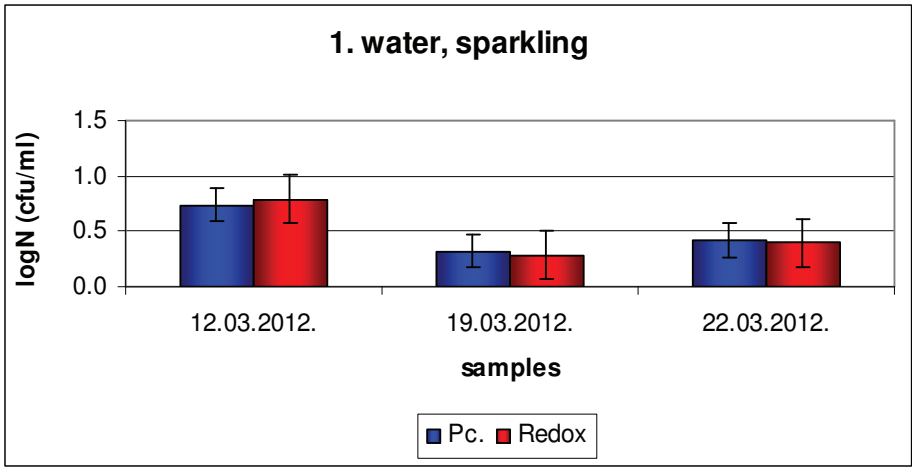

Figure 11. Microbial count result of the 1st water sample (lgN ± SD)

The Appropriate time required for the detection of the results using the Micro tester varied according to the LogN. The higher the LogN (cfu/ml) the lower the TTD time.
The TTD varied from 22-12 hours. We can say that the TTD and LogN are inversely proportional.
The Appropriate time required for the detection of the results using the classical pouring method was 72 hours.

6. CONCLUSION

The MicroTester that uses the redox potential measurement based method is applicable for determination of the microbial count of mineral waters as it can be carried out without the need for pre-enrichment for the microbes.

In opposite to the classical method, when we used 1 ml water for determination of the microbial count with plate pouring method, with the Micro tester equipment up to two liters can be evaluated.

There is also a significant difference between the classical method and the Micro Tester method in regards to the detection criteria. In the case of membrane filtering and plate pouring method to appear a single colony from 10^0 to 10^7 is needed. In the case of redox, from the initial cell concentration to the 10^6 cell/ml detectable cell concentration is needed.

The time required for the determination of the results is greatly reduced when using the MicroTester 17 hours on average, in comparison to the classical method which required 72 hours for determination of results.

This projects confirms that the MicroTester is suitable for the examination of the microbial content in mineral water as it enables a more rapid and reliable result in comparison to the classical culturing methods.

In regards to the water industry this is indispensable as it ensures the safety and prevention of Health risks from occurring.

7. SUMMARY

Mineral water comprises of 44% of the non alcoholic drinks market in Europe.
On average 105 litres of mineral water is consumed each year in Europe per capita.

There is a vast number of water borne pathogens that can be present in mineral water e.g. *E coli, Salmonella, Cryptosporidiosis, Giardia, Hepatitis.*

To ensure the protection of consumers the European Union enforced Commission Directive 2003/40/EC of 16 May 2003 establishing the list, concentration limits and labeling requirements for the constituents of natural mineral waters and the conditions for using ozone-enriched air for the treatment of natural mineral waters and spring waters.

The MicroTester system developed and patented by the researchers of the Department of Food Hygiene (Szent István University, Faculty of Veterinary Science) and Department of Physics and Automatization (Corvinus University, Faculty of Food Science) is based on the detection of change in redox-potential, caused by microbial activity. This method is suitable for significantly reducing the time requirement of microbiological testing.

Our present research shows that by the application of redox- potential measurements, we can significantly accelerate the detection of microbes in mineral water. The time requirement of the detection of the presence of microbes in mineral water by the redox potential measurement was 17 hours, by the classical method it was 72 hours.

A further advantage of the redox-potential measurement method is the investment cost is almost one third of that of the impedimetric methods and it allows the application of standard culture media.

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