GENOMIC BACTERIAL DIVERSITY AND QUALITY OF BOTTLED DRINKING WATER IN LEBANON

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ABSTRACT

GENOMIC BACTERIAL DIVERSITY AND QUALITY OF BOTTLED DRINKING WATER IN LEBANON

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Abstract

Lebanon is a country on the Mediterranean Sea with a mild Mediterranean climate, and abundance of fresh water falling on its mountains. Several problems face the water sector in Lebanon, with natural and human causes leading to the quantitative and qualitative deterioration of the water reserves. The lack of sewage systems and the lack of control on water quality has led to more and more people to use bottled waters as a source for drinking water. Bottled water companies in Lebanon use mainly springs located in the highest mountains of Lebanon. In this study the hygienic quality of bottled water was studied over a period of one year using tests for indicators of contamination such as coliforms, *Aeromonas spp., Pseudomonas spp., Enterococcus spp* . and total hetrotrophic bacterial counts. This study was carried out on the major brand names of Lebanese bottled water which are widely consumed in the market. These brands are Brand a, Brand F, Brand D, Brand C, Brand B and Brand E.

In this study 30.7% of recovered pseudomanads from tested samples where confirmed as *P. aeruginosa,* of the 15 isolated colonies on the *Aeromonas* medium only 4 were confirmed to be *Aeromonas sp.* (26.7%), faecal coliforms were not detected in any of the tested bottled drinking water samples and no enterococci were isolated from any brand.

Brand A, Brand B and Brand F all had a mean HPC (in all four batches) less than 500 cfu/ml, while the number was 677 cfu/ml in Brand E bottled water. However, Brand C and Brand D had mean HPC counts of 4022 cfu/ml and 4923 cfu/ml.

The identification of organisms using the Biolog and API systems in this study revealed that with 46% of the samples different results were obtained, while 44% were not identified using the Biolog system and only 10% gave a matching ID when compared to the ID obtained by using 16S rDNA sequencing. The 16S rDNA identification resolved the ambiguity observed with the Biolog identification system.

The overall microbial quality of Brand A and Brand F were the most acceptable and within all standards. The microbial quality of Brand B and Brand E were also acceptable although *Pseudomonas aeruginosa* was detected in both brands, while *Aeromonas sp.* CDC 715-84 was isolated from 75% of the tested Brand C bottles. The HPC count was very high and did not adhere to international or Lebanese standards in 50% of the tested batches of Brand C and in 100% of tested batches in Brand D. The identified microorganisms gave a fingerprint that showed that all tested brands had a fixed source throughout this study except Brand B which had a constantly changing source. Seasonal variation did not have any significant effect on water quality. Moreover,this study showed that results obtained using the Biolog system for the identification of environmental samples should be carefully interpreted. The quality of bottled drinking water in Lebanon should be properly monitored.

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1- General Introduction

Lebanon is a country on the Mediterranean Sea with a mild Mediterranean climate, and abundance of rain and snow falling on its mountains. Several problems face the water sector in Lebanon, with natural and human causes leading to the quantitative and qualitative deterioration of the water reserves (Khawlie, 2001). There are about 2500 major springs, and a large number of minor springs. In Lebanon a realistic estimation of water volume discharged from these springs is 1150 Mm^3/year. About 70% of Land in Lebanon is made of calcareous rocks, mainly fractured and cavernous limestone with the groundwater having a high concentration of calcium, carbonate and ferrous ions (Plassard, 1992). The fact that not all areas of Lebanon have public water distribution systems has led to the wide spread use of artesian wells in rural areas. The depth of these wells varies from 50 to 300 m depending on the aquifer with an average of about 150 m (Plassard, 1992). This depth combined with the lack of sewage systems and the lack of control on water quality has led to more and more people to use bottled waters as a source for drinking water.

In many developing countries, municipal water is unsafe because of inadequately maintained pipes, low pressure, intermittent delivery, and clandestine connections (Ford & Colwell, 1996), with possible consequences on the microbiological quality of the drinking water itself (Hashwa & Tokajian, 2000).

Bottled water companies in Lebanon use mainly springs located in the highest mountains of Lebanon for example Rim water is bottled from a spring at 1450 m above sea level (Rim Co.). Many consumers choose bottled water because they believe it

contains fewer contaminants then other sources and is a healthier choice (Dixon, 1988). Naturally groundwater is expected to be of excellent microbiological quality and generally of adequate chemical quality. This is due to the soil barrier providing effective isolation from surface pollutants (Foster, 1995). In recent years there has been a growing suspicion that the protection of groundwater quality is not always assured by a soil barrier and therefore the contamination of groundwater from different sources occurs worldwide, with heterotrophic bacteria and coliforms being detected in groundwater sources. Chilton et al. (1995) revealed that pathogens may survive in groundwater for up to 40 days given favorable conditions and reasonable levels of nutrients.

Bottled water is not sterile, with several chemical and physical treatments being carried out at the plant prior to bottling. The Environmental Protection Agency (EPA), World Health Organisation (WHO) and the International Bottled Water Association (IBWA) have all set standards and regulations for the production of safe bottled water. These standards are used as a legal framework by governments to ensure bottled water quality control. In Lebanon standards are set by the Ministry of Health and in general are stricter than all measures recommended by the three authorities previously listed (MOH, 1999). Although the Laws are in place but how closely the companies abide by them is the real issue that will be measured during the course of this study.

Bottled Water characterized by having an autochtonous microbiota, with allochtonous populations being introduced in some rare cases from the water itself, or during the bottling (Bischofberger et al., 1990). Bacteria introduced into the water could be important from a health perspective, especially in the presence of pathogenic

microorganisms, which could multiply and become persistent in the water (Morais et al., 1997)

To test for water contamination, indicators such as coliforms, *Aeromonas spp.*, *Pseudomonas spp.*, *Enterococcus spp.* and total hetrotrophic bacterial counts, could be used to determine feacal contamination (Morais, 1997). Some of these however, are known to be opportunistic pathogens, and their presence in drinking water is not desirable (Warburton, 1993). Therefore the quality of bottled water should be monitored and manufacturers should adhere to regulations set by the government and/ or specialised agencies such as EPA.

Many brands of bottled water label the bottles in a way to imply purity using terms such as "natural" or "pure". Sometimes however, the source is either not well identified, or the brand may be using more than one source (Weissman, 1997). An example is Nestle bottled water in Lebanon, the source is not identified and is made appealing by using the term "Pure Life" on the label. Whether this company uses the same spring as the Sohat Bottled Water, which it owns, or not is a question the consumer cannot realise and has to rely on the term "pure" as an indicator of water quality. The diversity within the microbiota when properly monitored could be an indication whether bottling conditions have changed or not and whether the source of water is constant or being changed during the course of bottling.

Bottled water quality is determined by regulating and determining the chemical and microbial composition, and this study will mainly focus on its microbial diversity. The microbial quality of bottled water was monitored during a period of one year to determine quality of the main brands on the market and correlate it with seasonal

changes. The international standards for bottled water include tests for coliforms, *Pseudomonas, Enterococci* and Total Hetrotrophic Plate counts (HPC), while in Lebanon we additionally have standards for anaerobic sulphate reducing bateria.

This study will be carried out on the major brand names of Lebanese bottled water which are widely consumed in the market. The bottle sizes studied will be those that are commonly available and used.

2-OBJECTIVES

- To determine the microbiological quality of Lebanese bottled water
- To compare bottled water quality to Lebanese and International standards
- To study the differences of bottled water quality during different seasons
- To study the microbiological diversity within the studied brands
- To compare the effectiveness of Phenotypic (Biolog) versus Genotypic (16S rDNA sequencing) identification of bacteria from bottled water

3- Literature Review

Microbiological quality of bottled waters has become a growing concern following the increase of bottled water consumption in the last decade. Bacteria introduced into bottled water could be important from a public health point of view if they are pathogenic or are persistent (Morais, 1997).

In Lebanon piped domestic water is supplied intermittently several times a week depending on local conditions. In urban areas 79 % of buildings are connected to safe drinking water whereas in rural areas the percentage is much lower (UN Resident Coordination System in Lebanon, 2001). Lack of appropriate control on the quality of water has led to widespread use of bottled water for drinking purposes in Lebanon, which is also the case in the rest of the world (IBWA, 2004).

3.1 Bottled Water Quality

Bottled water quality has to follow strict quality parameters. Both spring and mineral bottled water are tested and evaluated under the same legislations in most countries (Morais, 1997). It is common practice to determine bottled water quality through monitoring the levels of faecal indicators in water. Although some of these organisms are not pathogenic themselves, but could act as indicators reflecting faecal contamination (Dewittinck, 2001). Although water could be of good quality at the source, it might deteriorate through subsequent handling (Pip, 2000). The safety of bottled water could be monitored and safety insured by the use of Model Codes similar to the ones developed by the International Bottled Water Association (IBWA) which are a set of

standards and recommendations for the monitoring and controlling bottled water quality (IBWA, 2004). EPA has established standards, which in the USA are part of the Law and are as stringent as EPA' s standards for tap water (IBWA, 2004).

3.1.1 Coliforms in Water

Coliforms are a broad group of bacterial species present in abundance in the intestines of warm-blooded animals and are excreted in their feaces (Romprea et al., 2002). The coliforms as a group are very diverse. In Standard Methods for the Examination of Water and Wastewater they are described as: all aerobic and facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35 °C (APHA et al., 1998). However the definition has recently been extended to include other characteristics such as the test for galactosidase (APHA et al., 1998).

These bacteria are used as indicator organisms in monitoring water quality since their presence could imply faecal contamination. With *Escherichia coli* being strictly related to faecal contamination while other members of the group such as *Klebsiella, Enterobacter* and *Citrobacter* being predominant environmental organisms (Leclerc et al., 2002)

In a study done by McFeters et al. (1986) the comparative survival of organisms in well water indicated that coliform bacteria have a half-life of 17 hours, the pathogenic *Salmonella typhi* 6 hours and *Vibrio cholerae* 7.2 hours. With coliforms being effective indicators for the absence of these pathogens.

The United States EPA has indicated that the presence of total coliforms is a possible health concern and their detection in drinking water is important due to their

association with sewage and animal waste. According to the WHO, IBWA and EPA total coliform counts should be 0 per 100 mL in drinking water samples (WHO, 1984; IBWA, 2004). The Ministry of Health in Lebanon has set the limit also to 0 cfu/100 ml in bottled water (MOH, 1999). *E. coli* accordingly should not be detected in drinking water or bottled water. Traditionally the enumeration of coliforms is based on lactose fermentation, some other new methods are being employed such as testing for ß-glucuronidase activity with fluorogenic or chromogenic compounds (Neimi et al., 2001)

3.1.2 Pseudomonas in Water

Fluorescent *Pseudomonas* strains constitute a diverse group of bacteria that can be visually distinguished from other pseudomonads by their ability to produce a water soluble yellow-green pigment (Dabboussi et al., 1999). *Pseudomonas aeruginosa* is a ubiquitous environmental bacterium. It is an opportunistic pathogen that can cause community or hospital acquired (nosocomial) infections. Since *P. aeruginosa* is capable of growing in oligotrophic waters for extended periods of time and thus its occurrence in natural mineral water should be limited (Hardalo & Edberg, 1997). It can be isolated often in high numbers from food especially vegetables and from human or animal excrement. However its presence in faeces is not consistent, thus it cannot be used as a strict indicator for faecal pollution (HMSO, 1994). *P. aeruginosa* can survive and remain culturable for long periods compared to other Gram-negative bacteria (Byrd et al., 1991). *P. aeruginosa* should not be detected in a sample of 250 ml of bottled water (Morais, 1997), although some strains of fluorescent *Pseudomonas* were detected in samples from natural mineral waters by Elomari, et al. (1995). Hunter (1993) reported that 29 % of

Pseudomonas sp. strains isolated from bottled water were *Pseudomonas aeruginosa,* while Mania et al. (1990) detected *Pseudomonas sp.* in 83 % of batches of bottled water collected from retail outlets. In Lebanon standards controlling bottled water quality mandates that no *Pseudomonas aeruginosa* should be detected in any 250 ml sample of bottled water (MOH, 1999). This however is also the reccomendation by the IBWA and EPA although not considered as a binding standard (IBWA, 2004).

3.1.3 Aeromonas in Water

Aeromonas are widespread in surface waters and can therefore occasionally be detected in bottled water. The ability of *A. hydrophila* to grow in drinking water in the presence of low concentrations of organic compounds is well established (Kersters et al., 1996). Investigations have shown a high incidence of *Aeromonas spp.* in municipal drinking water supplies (Clark et al., 1982), which could survive standard chlorination and colonize chlorinated water distribution networks (van der Kooij, 1992);(Tokajian and Hashwa, 2004). *Aeromonas spp.* can cause gastroenteritis, wound infections, bacteremia, and miscellaneous other infections including endocarditis, meningitis, pneumonia, conjunctivitis, and osteomyelitis. A greater risk of infection is reported in young children, elderly people, and immuno-compromised patients (Janda, 1991). *A. hydrophila* has been increasingly recognized as an enteric pathogen and has been associated with a wide variety of human infections. The enteropathogenicity of *Aeromonas* spp. was attributed to the production of extoxins (Cahill, 1990). The presence of A*eromonas spp.* in drinking water is a potential risk since these organisms produce a wide range of virulence factors (Ivanova, 2001), with *A. caviae* and *A. sobria* being associated with enteritis (Janda et al.,

1996). Moreover, *A. schubertii*, *A. jandaei* and *A. veronii* being also isolated from drinking waters, are known to be human pathogens (Janda & Abbott, 1998). Several attempts have been made in some countries such as Italy and Canada, to introduce standards for A*eromonas spp.* in drinking water. In Italy standards were set for natural mineral waters at their origin at 10 colonies/100 ml and after being bottled at 100 colonies/100 ml (Villari, 2003). International standards for the number of A*eromonads* however, were not set neither by EPA nor IBWA, and accordingly it is also absent from the Lebanese standards (MOH, 1999).

3.1.4 Enterococci in Water

Groundwater represents about 40% of the water used for public water supplies and provides drinking water for more than 97% of the rural population in the United States. Recent monitoring studies have demonstrated the vulnerability of groundwater to contamination due to seepage from waste lagoons (Sanford, 2001). *Enterococci.* are important indicators of the quality of water and are known to be a leading cause of nosocomial infections (Jackson, 2004). The IBWA and EPA have not set a strict standard, but its recommended that no *Enterococcus* spp. should be detected in any 250 ml sample. In Lebanon the limit for *Enterococcus* spp. in bottled water is set to be 0 cfu per 250 ml (MOH, 1999)

3.1.5 Heterotrophic Plate Count (HPC) Bacteria

Determination of the heterotrophic plate count (HPC) bacteria in bottled water was found to be very useful in assessing water quality (Carter et al., 2000). The numbers

can be used as an indication of the water quality both during storage and distribution in the network (Carter et al., 2000). In a study conducted by Payment et al. (1991) the potential impact on human health following the consumption of treated water with high HPC levels was not clear. HPC bacteria may be useful in defining water quality. Pigmented bacteria are considered potentially useful markers for interpreting changes in the microbiological quality of finished water. Although pigmented bacteria generally go unnoticed due to their slow growth, they can be detected on R2A agar after being incubated for 7 days (Geldreich, 1996).

Reasoner and Geldreich (1985) suggested that R2A agar can be used for the cultivation of HPC bacteria isolated from drinking water. The spread plate technique was advocated for the recovery of oligotrophic organisms, were all plates should be incubated for not less than 72 hours and up to five to seven days at 35°C or a minimum of five days at 20 or 28°C (Hach, 2000). In a study performed by Geldreich (1996) on conventionally treated drinking water samples from a distribution network revealed that incubation at 20°C yielded the highest counts when incubation was to 12–14 days, whereas 28°C appeared to be the best temperature from day 2 through day 6 of incubation.

Although there has been no association between HPC bacteria and human health problems the IBWA and EPA have indicated that the HPC should not exceed 500 cfu per 1 ml at 28 °C after 5-7 days of incubation. On the other hand the Lebanese Ministry of Health has set a binding limit of 100 cfu per 1 ml at 22 °C after 3 days of incubation.

3.2 Bacterial Identification Phenotypic versus Genotypic Methods

The greatest barrier in obtaining information on HPC bacteria is the almost overwhelming task of identifying this diverse group of bacteria (LeChevallier, 1980). The traditional methods used for bacterial identification rely on phenotypic methods. Clinical microbiology has seen major advances in the development of microbial identification systems (Stager, 1992). Among the phenotypic methods the most common and widely used commercial identification systems are the API (bioMerieux, France) and the Biolog (Biolog inc., USA). The Biolog system relies on substrate utilization to obtain a fingerprint, which can be used for bacterial identification (Biolog inc., USA). The major problem with the available phenotypic methods is with slow growing and fastidous microorganisms, where interpreting results can involve a substantial amount of subjective judgement (Drancourt et al., 2000). Additionally some strains can exhibit phenotypic variability with atypical characteristics interfering with the identification (Drancourt et al., 2000).

The 16S rDNA sequence based identification of bacteria offers a useful alternative where phenotypic identification fails. Drancourt et al. (2000) found that the 16S rDNA method could identify 90% of 177 isolates that the API system failed to identify. Sequence analysis of the 16S rDNA represents a highly accurate and versatile system for bacterial classification and identification (Song et al., 2003; Weisburg *et al.*, 1991). Signature nucleotides of the 16S rDNA allow classification even if a particular sequence has no match in the database, which means otherwise unrecognizable isolates can be assigned to certain phylogentic branches (Song et al., 2003)

Recent advances in DNA sequencing technology greatly enhanced the identification of bacterial isolates and facilitated the shift from the use of classical techniques (such as substrate utilization based identifaction) to molecular techniques (Boye et al.,1999; Kolbert and Persing, 1999). DNA sequencing has several advantages especially that DNA can easily be extracted and can be used for routine analysis (Kolbert and Persing, 1999). The 16S is widely used because of its smaller size, while the 23S allows for a higher resolution (Brock, 1988; Woese, 1987). The widespread availability of published 16S rDNA sequences and availability of a wide range of databases makes 16S rDNA the ideal choice for definitive bacterial identification (Woese, 1987).

4-Materials and Methods

4.1 Water Samples

This work will include the analysis of bottled water samples taken from six major brand names in the Lebanese market and include Rim, Tannourine, Sohat, Nestle, Sannine and Sabil. Bottles corresponding to the volumes continuously available on the market were taken into consideration. The 1.5 liter bottles were used for Sannine, Rim and Tannourine, the 2 liter bottles for Nestle, the 500 ml bottles for Sohat and the 5 liter gallons for Sabil. Samples were tested in 4 batches according to the following scheme: Batch 1- Winter 2003/2004, Batch 2 - Spring 2004, Batch 3 - Summer 2004, Batch 4- Winter 2004

Every batch included a set of 3 bottles from each brand with the date of production being taken into consideration. All bottles were stored under the similar conditions between the date of acquisition and analysis which was 3-4 days.

4.2 Microbiological Parameters

4.2.1 Enumeration of *Pseudomonas*

To determine the number of pseudomonads in the drinking water samples the membrane filtration technique was used (HMSO, 1994). A 250 ml sample of bottled water was filtered using 0.45 μm pore-size Millipore membrane filters and Nalgene filtration apparatus. Filters were placed on (35 x 10 mm) sterile petri dishes containing Pseudomonas C-N Agar (PA) (Oxoid). The medium was prepared using Pseudomonas Agar base (Oxoid) supplemented with C-N (Oxoid), which contains cetrimide and sodium nalidixate. Tests were done in duplicates for every set of 3 bottles. Plates were

incubated at 30°C for 48 h. Plates were then examined under white and UV illumination. Colonies showing blue-green or brown pigmentation or fluorescence under the UV light were considered presumptive *Pseudomonas spp.* (HMSO, 1994). *Pseudomonas aeruginosa* normally appear as straw coloured colonies with green fluorescence, however tests should be conducted to identify the Pseudomonads at the species level. According to the Lebanese standards *Pseudomonas aeruginosa* should not be detected in a 250ml drinking water sample (MOH, 1999).

4.2.2 Enumeration of *Aeromonas*

To determine the presence and number of Aeromonads in the drinking water samples the membrane filtration technique was used (HMSO, 1994). A 250 ml sample of the bottled water was filtered using 0.45 μm pore-size Millipore membrane filters and Nalgene filtration apparatus. Filters were placed on (35 x 10 mm) sterile petri dishes containing Aeromonas medium base (Oxoid) supplemented with ampicillin (Oxoid). Tests were done in duplicates for every bottle in a set of 3. Plates were incubated at 30 °C for 24 h. Aeromonads form dark green colonies with dark centers and are 0.5-1.5 mm in diameter. According to Lebanese standards *Aeromonas* should not be detected in 250 ml of bottled water sample (MOH, 1999)

4.2.3 Enumeration of *Enterococci*

To determine the presence and the number of e*nterococci* in the drinking water samples the membrane filtration technique was used (HMSO, 1994). A 250 ml sample of the bottled water was filtered using 0.45 μm pore-size Millipore membrane filters and Nalgene filtration apparatus. Filters were placed on (35 x 10 mm) sterile petri dishes containing Enterococcosel agar (BBL) which is also known as bile esculin azide agar.

Tests were done in duplicates for a set of 3 bottles. Plates were incubated at 37 ° C for 24 h. *Enterococci* appear, as black opaque colonies with esculin hydrolysis meaning discoloration of agar under and around the colony. According to the Lebanese Standard *Enteroccocus* should not be detected in 250 ml bottled drinking water sample (MOH, 1999).

4.2.4 Enumeration of Coliforms

Coliforms were enumerated in the samples using the membrane filtration technique (HMSO, 1994). A 100 ml sample of the bottled water was filtered using 0.45μm pore-size Millipore membrane filters and Nalgene filtration apparatus. Filters were placed on (35 x 10 mm) sterile petri dishes containing lauryl sulfate agar (Oxoid) . Tests were done in duplicates for a set of 3 bottles. Plates were incubated at 28 ° C for 2 h and then for a further 24 h at 37°C. All yellow colonies representing presumptive coliforms were counted. According to the Lebanese standards coliforms should not be detected in a 100 ml sample of bottled drinking water (MOH, 1999).

4.2.5 Enumeration of Heterotrophic Bacteria

Heterotrophic bacteria were enumerated in the samples using the membrane filtration technique (HMSO, 1994). A 100 ml sample was filtered using 0.45 μm pore-size Millipore membrane filters and Nalgene filtration apparatus. Samples were first diluted 100 and 1000 x with cold ringer's solution (the composition of which is listed in appendix I) (HMSO, 1994), which was removed from the refrigerator immediately prior to filtration so as to minimize the effect of the solution on the viability of the bacteria.

Filters were placed on (35 x 10 mm) sterile petri dishes containing R2A agar (Oxoid) Tests were done in duplicates for a set of 3 bottles. Plates were incubated at 28 ° C for 5 days, the number and morphological characteristics of the colonies were determined. According to Lebanese standards heterotrophic plate count should not exceed 100 colonies/ml of bottled drinking water sample (MOH, 1999).

4.3 Enrichment and Purification of Bacteria

Colonies growing on any type of media were isolated and purified on Tryptose soy agar (TSA) medium except for HPC bacteria which were isolated on R2A medium. Plates were incubated at 30 ° C for presumptive *Aeromonas*, *Pseudomonas* and HPC and at 37 ° C for *Enterococcus* and coliforms. Plates were incubated 24-48 h. Pure colonies were used to determine Gram-reaction and oxidase activity using 0.1% w/v tetramethyl-p-phenylene diamine hydrochloride reagent.

4.4 Bacterial Identification Using the Biolog identification system

Identification of bacterial samples is considered to be one of the most important aspects of this study. The most widely used commercial phenotypic method is the Biolog system (Biolog inc., USA).

The Biolog GN2 microplates were used for the identification of the gram-negative bacteria and GP2 microplates for gram-positive bacteria.

4.4.1 Inoculom Preparation

Inocula for identification were prepared from overnight cultures on tryptone soy agar (Oxoid). Pure colonies were picked using a cotton swab and suspended in the inoculating fluid (IF). The Inoculating fluid (IF) was prepared using 0.01% Gellan Gum (Sigma), 0.04%NaCl and 0.03% Pluronic F-68 (Sigma). Tubes containing 20 ml of the inoculating fluid were sterilized prior to innoculation.

The turbidometer was calibrated using the IF as a blank. This was followed by the preparation of the bacterial suspensions with the turbidity being adjusted to the values recommended by the manufacturer for Gram-positive and negative bacteria. All prepared inocula were used within 10 minutes to minimize any changes in the viability of the bacterial cells. Microplates were inoculated by adding 150 μl of inoculating fluid in each well of the plate using an eight-channel multipipette.

Microplates were incubated at 28°C for 24 h. Changes in the colour of the wells were detected using the Biolog microplate reader. Identification based on the metabolic profiles was conducted using the Microlog software (version 4.01B).

4.5 Bacterial Identification Using 16S rDNA gene Sequencing

4.5.1 DNA Extraction

Fresh overnight pure bacterial cultures were used for DNA extraction. DNA was extracted from 4-5 bacterial colonies using the Nucleospin extraction kit (Macherey-

Nagel) following the manufacturers instructions and using the protocol for bacterial DNA extraction.

4.5.2 16S rDNA Amplification

16S rDNA was amplified using the 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T-3') universal primers (Thermo Hybaid, Germany) (Suzuki and Giovannoni,1996)

The following were combined in a 0.2 ml MicroAmp PCR tube: 0.3µl of (25mM) premixed deoxynucleoside triphosphates (dNTPs) (ABGene), 2µl of (25 mM) $MgCl_2$, 2µl of 10 x buffer (ABGene), 0.2 µl (5U/µl) Taq polymerase (ABGene), 1 µl of extracted DNA, and 0.2 µl of (50 pmol/µl) forward and reverse primer mix and 14.3 µl sterile distilled water. Cycle amplification was performed on a Perkin Elmer GeneAmp 9700 thermal cycler (Cycle amplification: 1 cycle at 2 mins 95°C; 30 cycles of 30 sec at 94°C, 30 sec at 46°C and 2 min at 72°C and a final soak at 4°C).

4.5.3 PCR Products purification

PCR has in addition to the amplified fragment, primer dimmers, unused DNTPs and the *Taq polymerase* enzyme. Accordingly, DNA was purified prior to sequencing using Ultra PCR Clean-Up Kit (ABGene, UK). Manufacturer's instructions were followed and 30 µl of the elution buffer instead of 50 µl was used to increase yield.

The DNA was visualised using standard agarose gel electrophoresis and ethidium bromide (Sambrook et al, 1989).

4.6 Sequencing of 16S rDNA and Genotypic Identification

Sequencing was conducted using the AlfExpress system (Amersham Biosciences), the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit and the Alfwin analysis software.

4.6.1 PCR products sequencing

The PCR Products were sequenced using Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit, which contains all the needed components in addition to a universal primer. The universal primer was substituted by the 27F primer (section 4.5.2) (Thermo Hybaid,Germany). Cy5 ddNTP's provided were handled in the dark, as they were very light sensitive. Only 1µl of the purified DNA template was used in the reaction mix and cycle sequencing was performed on a GeneAmp 9700 Thermal cycler (cycle sequencing conditions were: 40 cycles at 95° C for 30 sec, 60° C for 30 sec, and 72 ° C for 80 sec).

4.6.2 Ethanol Precipitation

Following thermal cycling excess dye terminators were removed by ethanol precipitation as recommended by the sequencing kit manufacturer. The contents of each PCR tube were pipetted into a tube containing 2 µl of 7.5 M ammonium acetate, 2 µl glycogen solution and 30 µl chilled 100% ethanol. Tubes were mixed thoroughly and incubated overnight at -20°C, followed by centrifugation at maximum speed (Eppendorf Microcentrifuge, 5415D) for 30 min. Supernatant was carefully removed and washed with 200 µl of chilled 70% ethanol, followed again by centrifugation for 5 mins at

maximum speed. The supernatant was removed and tubes were incubated at 70°C for 2 min to remove any excess ethanol in a thermomixer (Eppendorf Thermomixer).

Prior to loading samples onto the gel the pellet was resuspended in 6 µl of formamide loading dye and vortexed for 2-3 sec. Then heated to 70°C for 2-3 min and cooled immediately on ice. All the mixture was then loaded into the designated well on the gel.

4.6.3 Sample Analysis

The Gel used was the Reprogel High Resolution (Amersham biosciences) prepared according to manufacturer's instruction. The Gel was loaded into a short cassette provided with the Alfexpress unit and the gel thickness was set to be 0.5 mm. The gel was run using 2 litres of 0.5 x TBE buffer and under the following settings 300 minutes, 1500 volts, 60 mA, 25 W, 55 °C and at a sampling interval of 1 sec. The software used to read and analyze the results was the Alfwin Sequence Analyser version 2.11. The Data was processed using the shift recommened by the kit being used which was: A:-20 C:10 G:-5 T:15

Once the data were processed and ambiguities minimized by manual manipulation, the sequences were then compared using Blast Direct 16S rDNA sequences comparison was conducted using the EMBL (FASTA version 3.3t09)(Pearson and Lipman, 1988). Phylogenetic trees were constructed using the PHYLIP software (Felsenstein, 2005) which implements the substitution rate calibration method. This method considers the substitution rates of the individual nucleotides in a sequence alignement in the computation of of evolutionary distances. The trees were used to

determine the identification of isolates by calculating the closeness of an isolates DNA sequence with the sequence of known organisms in the EMBL database.

4.7 Statistical analysis

Statistical analysis was done to study changes in the quality throughout the year. The standard deviation curves were drawn using the Microsoft Excel software.

5 Results:

A table Summarizing all the quantitative results can be found in appendix I

5.1 *Pseudomonas aeruginosa* in Bottled Water

Presumptive *Pseudomonas* colonies were isolated on Pseudomonas Agar Base supplemented with cetrimide and sodium nalidixate. All colonies that were green, brown, orange or showed fluorescence were considered as presumptive Pseudomonads. Colonies were assigned designated as JM-PS# with those having different morphologies being given different numbers. By Lebanese standards *P. aeruginosa* should not be detected from any 250 ml sample of bottled water (MOH, 1999).

Presumptive pseudomonad colonies were detected only in one of the Brand A tested batches (Table 1), with two other different types of colonies detected in batch 3 with a mean colony count of 15 / 250ml, while in the Brand B brand presumptive pseudomonad colonies were detected in batch 1 (22/ 250ml) and batch 3 (1/ 250ml) (Table 2). In contrast, a high number of presumptive Pseudomonads were isolated in batch 1 of Brand C water (82 colonies/250 ml)(Table 3), while only 7 colonies were isolated from batch 2. Presumptive colonies were also recovered from batch 1 and 2 of the Brand E brand (Table 4), while none were recovered from neither Brand F nor Brand D brands. All isolated colonies were later identified using the Biolog and 16S rDNA identification methods.

Table 1: Number of presumptive pseudomonads in Brand A Bottled water

		Number of presumptive Pseudomonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand A	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	12(JM-PS1) + 3 (JM-PS2) = 15 colonies	11(JM-PS1) + 2 (JM-PS2) = 13 colonies	12(JM-PS1) + 5 (JM-PS2) = 17 colonies	15 colonies
	Batch4	0	0	0	0

Dates of production of each batch of Brand A water:
Batch1 (winter): 05/02/2004, batch2 (spring): 11/04/2004, batch3 (summer): 01/07/2004, batch 4: 29/11/04

Table 2: Number of presumptive pseudomonads in Brand B Bottled water

		Number of presumptive Pseudomonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand B	Batch1	22 (JM-PS3)	23 (JM-PS3)	22 (JM-PS3)	22 colonies
	Batch2	0	0	0	0
	Batch3	0	3 (JM-PS4)	1 (JM-PS4)	1 colony
	Batch4	0	0	0	0

Dates of production of batches of Brand B water:
Batch1 (winter): 04/03/2004, batch2 (spring): 25/05/2004, batch3 (summer): 25/9/2004, batch 4 (winter): 14/11/2004

Table 3: Number of presumptive pseudomonads in Brand C Bottled water

		Number of presumtive Pseudomonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand C	Batch1	82 (JM-PS5)	82 (JM-PS5)	83 (JM-PS5)	82 colonies
	Batch2	4 (JM-PS6)	10 (JM-PS6)	8 (JM-PS6)	7 colonies
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Dates of production of batches of Brand C water:
Batch1 (winter): 09/03/2004, batch2 (spring): 20/05/2004, batch3 (summer): 22/9/2004, batch 4 (winter): 11/11/2004

Table 4: Number of presumptive pseudomonads in Brand E Bottled water

		Number of presumptive Pseudomonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand E	Batch1	40 (JM-PS7)	48 (JM-PS7)	48 (JM-PS7)	42 colonies
	Batch2	20 (JM-PS8)	24 (JM-PS8)	16 (JM-PS8)	20 colonies
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Batch1 (winter): 01/12/2003, batch2 (spring): 05/05/2004, batch3 (summer): 12/07/2004, batch 4 (winter): 26/11/2004

5.2 *Aeromonas sp.* in Bottled Water

Presumptive *Aeromonas* colonies were isolated on Aeromonas medium Base (Oxoid) supplemented with ampicillin (Oxoid) also known as RYAN medium. All colonies that were dark green having dark centers with a diameter of 0.5-1.5 mm were considered as presumptive Aeromonads. Colonies were designated as JM-AE# with different numbers being given based on morphological differences. No standard is set for the presence of *Aeromonas* in bottled water by the Lebanese government.

Only one of the four batches of Bottled water from the Brand A brand showed presumptive *Aeromonas* colonies (Table 5). Two different types of colonies were isolated from batch 3 and the mean colony number was 43 colonies/ 250ml. Table 6 shows that batch1 of the Brand B brand of water had presumptive aeromonads. In batch 1 the number of isolated colonies was 111 colonies/250 ml distributed between two types of colonies based on morphological differences. However, a large number of presumptive aeromonads were recovered from all batches of Brand C bottled water with batch 1 having the highest number (721 colonies/ 250ml). In the other tested batches however, the numbers decreased to 100 colonies/ 250ml for batch 2, 18 colonies/ 250ml for batch 3 and 8 colonies/ 250ml for batch 4 (Table 7). Moreover, in Brand D bottled water the numbers fluctuated between a maximum of 972 colonies/ 250ml in batch 4 and a minimum of 60 colonies/ 250ml in batch 1 (Table 8). Two other different colonies again

based on morphological characteristics were recovered from batch 2 of Brand D bottled water, while in Brand E bottled water only 1 colony/ 250ml from one single batch was recovered (Table 9), while none were recovered from Brand F bottled water. All isolated colonies were later identified using the Biolog and 16S rDNA identification methods.

Table 5: Number of presumptive aeromonads in Brand A Bottled water

		Number of presumptive Aeromonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand A	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	10(JM-AE9) + 35(JM-AE10)= 45 colonies	10 (JM-AE9) + 33(JM-AE10)= 43 colonies	9 (JM-AE9) + 33(JM-AE10)= 41 colonies	43 colonies
	Batch4	0	0	0	0

Dates of production of each batch of Brand A water:
Batch1 (winter): 05/02/2004, batch2 (spring): 11/04/2004, batch3 (summer): 01/07/2004, batch 4: 29/11/04

Table 6: Number of presumptive aeromonads in Brand B Bottled water

		Number of presumptive Aeromonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand B	Batch1	6(JM-AE11) + 100(JM-AE12)= 106 colonies	6 (JM-AE11) + 109(JM-AE12)= 115colonies	8 (JM-AE11) + 105(JM-AE12)= 113 colonies	111 colonies
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Dates of production of batches of Brand B water:
Batch1 (winter): 04/03/2004, batch2 (spring): 25/05/2004, batch3 (summer): 25/9/2004, batch 4 (winter): 14/11/2004

Table 7: Number of presumptive aeromonads in Brand C Bottled water

		Number of presumptive Aeromonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand C	Batch1	722 (JM-AE13)	722 (JM-AE13)	718 (JM-AE13)	721 colonies
	Batch2	7 (JM-AE14) + 88 (JM-AE15) = 95 colonies	10(JM-AE14)+ 93(JM-AE15) = 103 colonies	10(JM-AE14)+ 92(JM-AE15)= 102 colonies	100 colonies
	Batch3	18 (JM-AE16)	17 (JM-AE16)	18 (JM-AE16)	18 colonies
	Batch4	7 (JM-AE17)	8 (JM-AE17)	8 (JM-AE17)	8 colonies

Dates of production of batches of Brand C water:
Batch1 (winter): 09/03/2004, batch2 (spring): 20/05/2004, batch3 (summer): 22/9/2004, batch 4 (winter): 11/11/2004

Table 8: Number of presumptive aeromonads in Brand D Bottled water

		Number of presumptive Aeromonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand D	Batch1	60 (JM-AE18)	60 (JM-AE18)	60 (JM-AE18)	60 colonies
	Batch2	48(JM-AE19)+ 725(JM-AE20) = 773colonies	45(JM-AE19)+ 725(JM-AE20)= 770 colonies	45(JM-AE19)+ 720(JM-AE20)= 765 colonies	769 colonies
	Batch3	360 (JM-AE21)	362 (JM-AE21)	360 (JM-AE21)	361 colonies
	Batch4	968 (JM-AE22)	975 (JM-AE22)	974 (JM-AE22)	972 colonies

Dates of production of batches of Brand D water:
Batch1 (winter): 07/03/2004, batch2 (spring): 17/05/2004, batch3 (summer): 24/9/2004, batch 4 (winter): 29/11/2004

Table 9: Number of presumptive aeromonads in Brand E Bottled water

		Number of presumptive Aeromonas colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand E	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	1 (JM-AE23)	0	1 (JM-AE23)	1 colony

Batch1 (winter): 01/12/2003, batch2 (spring): 05/05/2004, batch3 (summer): 12/07/2004, batch 4 (winter): 26/11/2004.

5.3 *Enterococcus* sp. in Bottled Water

Presumptive enterococci were isolated on enterococcosel agar (Oxoid). All colonies that were black and opaque and presented esculin hydrolysis were identified as presumptive enterococci. Colonies were designated as JM-EN#, with different morphologies being assigned different numbers. No *Enterococci* should be detected in any 250ml tested bottled water sample according to Lebanese standards (MOH, 1999)

In Brand A bottled water only 2 creamy white colonies grew on the enterococcus differential medium (Table 10). Although none were considered as presumptive enterococci they were kept for later identification. The same applies for Brand B bottled water (Table 11), while none of the other brands showed growth on this type of medium.

Table 10: Number of presumptive *Enterococcus sp,* colonies in Brand A Bottled water

		Number of colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand A	Batch1	1 (JM-EN24)	1 (JM-EN24)	1 (JM-EN24)	1 colony
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	4 (JM-EN25)	3 (JM-EN25)	4 (JM-EN25)	4 colonies

Dates of production of each batch of Brand A water:
Batch1 (winter): 05/02/2004, batch2 (spring): 11/04/2004, batch3 (summer): 01/07/2004, batch 4: 29/11/04

Table 11: Number of presumptive *Enterococcus sp.* colonies in Brand B Bottled water

		Number of colonies per 250 ml filtrate			
		First test	Second test	Third test	Mean
Brand B	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	2 (JM-EN26)	3 (JM-EN26)	3 (JM-EN26)	3 colonies

Dates of production of batches of Brand B water:
Batch1 (winter): 04/03/2004, batch2 (spring): 25/05/2004, batch3 (summer): 25/9/2004, batch 4 (winter): 14/11/2004

5.4 Coliforms in Bottled Water

5.4.1 Total coliforms tested at 30 ° C

Presumptive coliforms were isolated on lauryl tryptose agar (Oxoid). All yellow brown or orange colonies were considered as presumptive coliforms. Colonies were designated as JM-CM#, with colonies having different morphologies being assigned different numbers. According to the Lebanese standards total coliforms should not be detected in any 100 ml sample of bottled drinking water (MOH, 1999).

Total presumptive coliforms were only detected in one of the four batches of the Brand A brand. One type of colony based on morphological characteristics was isolated from batch 3 and the mean colony number was 2 cfu/ 100ml (Table12). Total presumptive coliforms were also detected in batch 1 of Brand B brand (Table 13), batch 1 of Brand C (Table 14), batch 3 of Brand D (Table 15) and batch 1 of Brand E bottled water (Table 16), with none being detected in Brand F. All isolated colonies were later identified using the Biolog and 16S rDNA identification methods.

5.4.2 Faecal Coliforms (Tested at 44.5 ° C)

Presumptive faecal coliform, were isolated on lauryl tryptose agar (Oxoid). All yellow brown or orange colonies were considered as presumptive coliforms. Colonies were assigned different numbers in the form of JM-CT#, with those having different

morphologies being assigned different numbers. According to the Lebanese standards coliforms should not be detected in a 100 ml of tested bottled drinking water (MOH, 1999). Batch 3 of Brand E bottled water had 4 cfu/ 100ml of presumptive faecal coliforms (Table 17), while none were recovered from any other brand.

Table 12: Number of presumptive coliform colonies in Brand A Bottled water

		Number of presumptive coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand A	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	2 (JM-CM27)	2 (JM-CM27)	3 (JM-CM27)	2 cfu
	Batch4	0	0	0	0

Dates of production of each batch of Brand A water:
Batch1 (winter): 05/02/2004, batch2 (spring): 11/04/2004, batch3 (summer): 01/07/2004, batch 4: 29/11/04

Table 13: Number of presumptive coliform colonies in Brand B Bottled water

		Number of presumptive coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand B	Batch1	1 (JM-CM28)	1 (JM-CM28)	0	1 cfu
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Dates of production of batches of Brand B water:
Batch1 (winter): 04/03/2004, batch2 (spring): 25/05/2004, batch3 (summer): 25/9/2004, batch 4 (winter): 14/11/2004

Table 14: Number of presumptive coliform colonies in Brand C Bottled water

		Number of presumptive coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand C	Batch1	1 (JM-CM29)	2 (JM-CM29)	1 (JM-CM29)	1 cfu
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Dates of production of batches of Brand C water:
Batch1 (winter): 09/03/2004, batch2 (spring): 20/05/2004, batch3 (summer): 22/9/2004, batch 4 (winter): 11/11/2004

Table 15: Number of presumptive coliform colonies in Brand D Bottled water

		Number of presumptive coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand D	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	2 (JM-CM30)	2 (JM-CM30)	2 (JM-CM30)	2 cfu
	Batch4	0	0	0	0

Dates of production of batches of Brand D water:
Batch1 (winter): 07/03/2004, batch2 (spring): 17/05/2004, batch3 (summer): 24/9/2004, batch 4 (winter): 29/11/2004

Table 16: Number of presumptive coliform colonies in Brand E Bottled water

		Number of presumptive coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand E	Batch1	1 (JM-CM31)	1 (JM-CM31)	1 (JM-CM31)	1 cfu
	Batch2	0	0	0	0
	Batch3	0	0	0	0
	Batch4	0	0	0	0

Batch1 (winter): 01/12/2003, batch2 (spring): 05/05/2004, batch3 (summer): 12/07/2004, batch 4 (winter): 26/11/2004

Table 17: Number of suspect coliforms isolated at 44.5 ° C

		Number of suspect coliform colonies per 100 ml filtrate			
		First test	Second test	Third test	Mean
Brand E	Batch1	0	0	0	0
	Batch2	0	0	0	0
	Batch3	3 (JM-CT32)	5 (JM-CT32)	3 (JM-CT32)	4 cfu
	Batch4	0	0	0	0

Batch1 (winter): 01/12/2003, batch2 (spring): 05/05/2004, batch3 (summer): 12/07/2004, batch 4 (winter): 26/11/2004

5.5 Determination of the Heterotrophic Plate Count (HPC/ml)

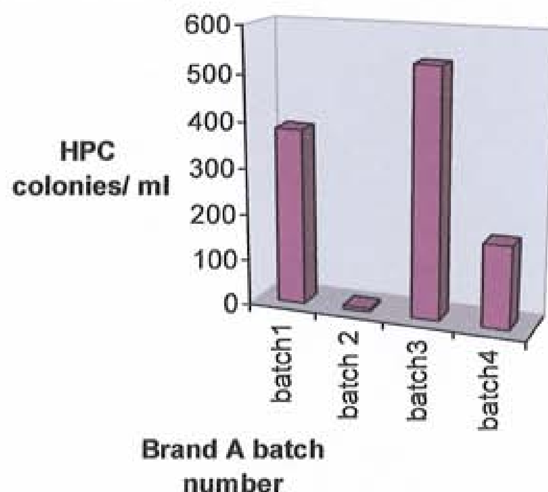
HPC counts were determined using R2A agar. Colonies on R2A agar were counted and designated as JM-H#, with different numbers being assigned for colonies showing morphological differences. The total HPC was then determined as the total number of colonies on the R2A agar. The Lebanese standard is 100 cfu/ ml for HPC (MOH, 1999) whereas the international standard is 500 cfu/ ml. The HPC values for Brand A water and the number of each representative colony are shown in Table 18 and Figure 1. The overall mean for all the batches was 275 cfu/ ml. The highest value was for batch 3, which was 533 cfu/ ml and the minimum value was for batch 2 (7 cfu/ ml). The overall mean for the Brand B bottled water was lower (165 cfu/ ml), with the highest number being detected in batch 2 (442cfu/ ml) and the minimum in batch 3 (Table 19, Figure 2). In contrast to the former brands the mean colony number was much higher in Brand C bottled water (4022 cfu/ml), with the highest value being detected in batch 2 (9617 cfu/ml) and the minimum in batch 4 (456 cfu/ml) (Table 20, Figure3). Similarly, high numbers of colonies were also recovered from Brand D bottled water (overall mean 4923 cfu/ml), with the maximum detected in batch 4 (5849 cfu/ml) and the minimum in batch 1 (3899 cfu/ml) (Table 21, Figure 4). Finally the mean colony number was 677cfu/ml for Brand E bottled water (Table 22, Figure 5), and was 677cfu/ml for Brand E bottled water (Table 22, Figure 5), and was 19 cfu/ml for Brand F bottled water since the value for batch 2 was omitted from statistical calculation due to its unusual high value in comparison to all other batches (Table 23, Figure 6)

5.5.1 HPC for Brand A Water

Table18: Number of HPC colonies/ml for Brand A

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand A	Batch1	**382 cfu** (JM-HS33)	**382 cfu** (JM-HS33)	**381 cfu** (JM-HS33)	**382 cfu/ml**
	Batch2	2 (JM-HS34) + 1 (JM-HS35) + 4 (JM-HS36) = **7 cfu**	3 (JM-HS34)+ 1 (JM-HS35) + 4 (JM-HS36) = **8 cfu**	3 (JM-HS34)+ 1 (JM-HS35)+ 3 (JM-HS36)= **7 cfu**	**7 cfu/ml**
	Batch3	13 (JM-HS 37) + 520 (JM-HS38) = **533 cfu**	12 (JM-HS37)+ 521 (JM-HS38)= **533 cfu**	12 (JM-HS37) + 521 (JM-HS38)= **533 cfu**	**533 cfu/ml**
	Batch4	18 (JM-HS39) + 3 (JM-HS40) + 160 (JM-HS41) = **181 cfu**	18 (JM-HS39) + 3 (JM-HS40) + 155 (JM-HS41) = **176 cfu**	17 (JM-HS39) + 2 (JM-HS40) + 163 (JM-HS41)= **182 cfu**	**179 cfu/ml**
				Mean	**275 cfu/ml**
				Standard Deviation	**199.4**

Figure 1 Chart to show the comparison of the HPC between the different tested batches



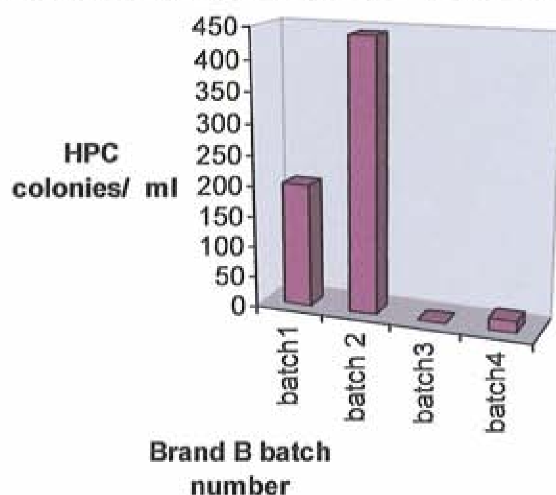
Dates of production of each batch of Brand A water:
Batch1 (winter): 05/02/2004, batch2 (spring): 11/04/2004, batch3 (summer): 01/07/2004, batch 4: 29/11/04

5.5.2 HPC for Brand B Water

Table19: Number of HPC colonies/ml for Brand B

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand B	Batch1	180 (JM-HN42)+ 20 (JM-HN43)= **200 cfu**	180 (JM-HN42) + 20 (JM-HN43) = **200 cfu**	181(JM-HN42) + 18 (JM-HN43) = **199 cfu**	**200 cfu/ml**
	Batch2	2 (JM-HN44) + 420(JM-HN45) = **422 cfu**	3 (JM-HN44) + 460(JM-HN45) = **463 cfu**	2 (JM-HN44) + 440(JM-HN45) = **442 cfu**	**442 cfu/ml**
	Batch3	**0**	**0**	**0**	**0**
	Batch4	**18 cfu** (JM-HN46)	**17 cfu** (JM-HN46)	**18 cfu** (JM-HN46)	**18 cfu/ml**
				Mean	**165 cfu/ml**
				Standard Deviation	**157.4**

Figure 2 Chart to show the comparison of the HPC between the different tested batches



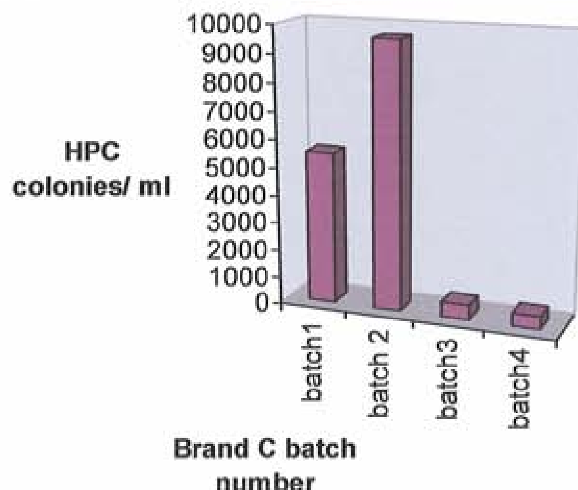
Dates of production of batches of Brand B water:
Batch1 (winter): 04/03/2004, batch2 (spring): 25/05/2004, batch3 (summer): 25/9/2004, batch 4 (winter): 14/11/2004

5.5.3 HPC for Brand C Water

Table20: Number of HPC colonies/ml for Brand C

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand C	Batch1	**5400 cfu** (JM-HR47)	**5640 cfu** (JM-HR47)	**5450 cfu** (JM-HR 47)	**5496 cfu/ml**
	Batch2	**9600 cfu** (JM-HR48)	**9650 cfu** (JM-HR48)	**9600 cfu** (JM-HR48)	**9617 cfu/ml**
	Batch3	80 (JM-HR49) + 440 (JM-HR50) = **520 cfu**	76 (JM-HR49) + 445 (JM-HR50) = **521 cfu**	75 (JM-HR49) + 443(JM-HR50) = **518 cfu**	**520 cfu/ml**
	Batch4	**430 cfu** (JM-HR51)	**472 cfu** (JM-HR51)	**465 cfu** (JM-HR51)	**456 cfu/ml**
				Mean	**4022 cfu/ml**
				Standard Deviation	**3822.9**

Figure 3 Chart to show the comparison of the HPC between the different tested batches



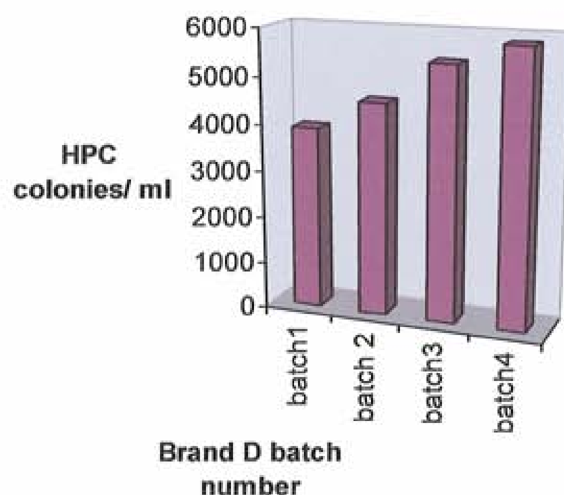
Dates of production of batches of Brand C water:
Batch1 (winter): 09/03/2004, batch2 (spring): 20/05/2004, batch3 (summer): 22/9/2004, batch 4 (winter): 11/11/2004

5.5.4 HPC for Brand D Water

Table21: Number of HPC colonies/ml for Brand D

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand D	Batch1	3600 (JM-HT52) + 250 (JM-HT53) + 30 (JM-HT54) = **3880 cfu**	3650 (JM-HT52) + 260 (JM-HT53) + 30 (JM-HT54) = **3940 cfu**	3600 (JM-HT52) + 250 (JM-HT53) + 28 (JM-HT54) = **3878 cfu**	**3899 cfu/ml**
	Batch2	**4540 cfu** (JM-HT55)	**4550 cfu** (JM-HT55)	**4500 cfu** (JM-HT55)	**4530 cfu/ml**
	Batch3	5410 (JM-HT56) + 9 (JM-HT57) = **5419 cfu**	5400 (JM-HT56) + 10 (JM-HT57) = **5410 cfu**	5400 (JM-HT56) + 10 (JM-HT57) = **5410 cfu**	**5413 cfu/ml**
	Batch4	5800 (JM-HT58) + 15 (JM-HT59) = **5815 cfu**	5850 (JM-HT58) + 17 (JM-HT59) = **5867 cfu**	5850 (JM-HT58) + 14 (JM-HT59) = **5864 cfu**	**5849 cfu/ml**
				Mean	**4923 cfu/ml**
				Standard Deviation	**758.4**

Figure 4 Chart to show the comparison of the HPC between the different tested batches



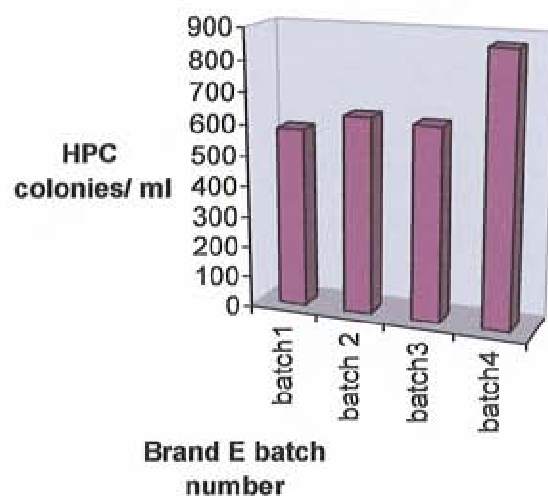
Dates of production of batches of Brand D water:
Batch1 (winter): 07/03/2004, batch2 (spring): 17/05/2004, batch3 (summer): 24/9/2004, batch 4 (winter): 29/11/2004

5.5.5 HPC for Brand E Water

Table22: Number of HPC colonies/ml for Brand E

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand E	Batch1	410 (JM-HSB60) + 180 (JM-HSB61) = **590 cfu**	400 (JM-HSB60) + 175 (JM-HSB61) = **575 cfu**	400 (JM-HSB60) + 178 (JM-HSB61) = **578 cfu**	**581 cfu/ml**
	Batch2	40 (JM-HSB62) + 580 (JM-HSB63) = **620 cfu**	42 (JM-HSB62) + 600 (JM-HSB63) = **642 cfu**	40 (JM-HSB62) + 600 (JM-HSB63) = **640 cfu**	**634 cfu/ml**
	Batch3	27(JM-HSB64) + 590 (JM-HSB65)= **617 cfu**	26 (JM-HSB64) + 600 (JM-HSB65) = **626 cfu**	27 (JM-HSB64) + 600 (JM-HSB65) = **627 cfu**	**623 cfu/ml**
	Batch4	**870 cfu** (JM-HSB 66)	**870 cfu** (JM-HSB66)	**870 cfu** (JM-HSB66)	**870 cfu/ml**
				Mean	**677 cfu/ml**
				Standard Deviation	**113.2**

Figure 5 Chart to show the comparison of the HPC between the different tested batches



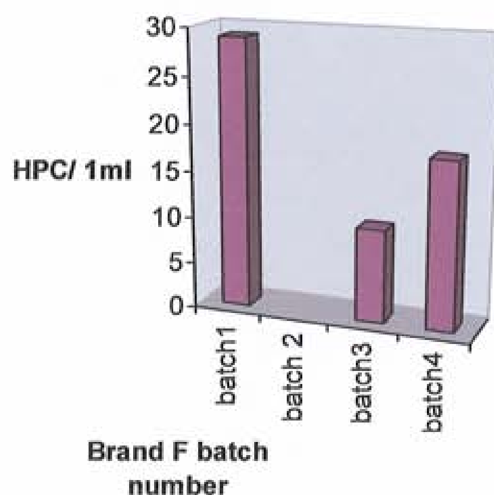
Batch1 (winter): 01/12/2003, batch2 (spring): 05/05/2004, batch3 (summer): 12/07/2004, batch 4 (winter): 26/11/2004.

5.5.6 HPC for Brand F Water

Table23: Number of HPC colonies/ml for Brand F

		HPC colonies/ml			
		First test	Second test	Third test	Mean
Brand F	Batch1	**28 cfu** (JM-HSN67)	**30 cfu** (JM-HSN67)	**30 cfu** (JM-HSN67)	**29 cfu/ml**
	Batch2	**515 cfu** (JM-HSN68)	**517 cfu** (JM-HSN68)	**520 cfu** (JM-HSN68)	**517 cfu/ml**
	Batch3	3 (JM-HSN69)+ 8 (JM-HSN70) = **11 cfu**	2 (JM-HSN69) + 8 (JM-HSN70) = **10 cfu**	3 (JM-HSN69) + 7 (JM-HSN70) = **10 cfu**	**10 cfu/ml**
	Batch4	15 (JM-HSN71) + 2 (JM-HSN72) + 2 (JM-HSN73) = **19 cfu**	14 (JM-HSN71) + 2 (JM-HSN72) + 1 (JM-HSN73) = **17 cfu**	15 (JM-HSN71)+ 3 (JM-HSN72) + 1 (JM-HSN73) = **19 cfu**	**18 cfu/ml**
				Mean	**19 cfu/ml**
				Standard Deviation	9.54

Figure 6 Chart to show the comparison of the HPC between the different tested batches



Batch1 (winter): 11/2/2004, batch2 (spring): 17/06/2004, batch3 (summer): 11/09/2004, batch 4 (winter): 26/11/2004.

5.6 Sample Identification
Phenotypic versus Genotypic

Two trials were done using the Biolog system and were designated as Id #1 and Id#2

5.6.1 Isolated colonies belonging to *α-proteobacteria*

Images of the isolated colonies can be seen in figure 7 and 8
Sphingomonas sp. strain DhA-33 (JM-AE 11), *Sphingomonas adhaesiva* (JM-AE 20) and *Sphingomonas aquatilis* (JM-HS 33) all of which are yellow smooth colonies and are examples of Sphingomonads. *Calobacter crescentus* (JM-HS 41) was observed as white creamy colonies (Figure 7).

Table 24: shows the isolated and identified colonies belonging to *α-proteobacteria* based on the use of the Biolog system and 16S rDNA sequencing.

α-proteobacteria	**Biolog Id #1**	**Biolog Id #2**	**16S rDNA sequencing Id.**
JM-AE 9	*Agrobacterium tumefaciens (95%)*	*Agrobacterium tumefaciens (95%)*	*Sphingomonas* strain DhA-33 (97%)
JM-AE 10	NO ID	NO ID	*Sphingomonas adhaesiva* (95.6%)
JM-AE 11	*Agrobacterium tumefaciens (96%)*	*Agrobacterium tumefaciens (95%)*	*Sphingomonas sp.* strain DhA-33 (97%)
JM-AE 12	NO ID	NO ID	*Sphingomonas adhaesiva* (95.5%)
JM-AE 13	NO ID	NO ID	*Sphingomonas adhaesiva* (95.6%)
JM-AE 15	NO ID	NO ID	*Sphingomonas adhaesiva* (95.6%)
JM-AE 18	*Agrobacterium tumefaciens* (98%)	*Agrobacterium tumefaciens* (98%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-AE 20	NO ID	NO ID	*Sphingomonas adhaesiva* (95.7%)
JM-AE 21	NO ID	NO ID	*Sphingomonas adhaesiva* (95.7%)
JM-AE 22	NO ID	NO ID	*Sphingomonas adhaesiva* (96%)
JM-AE 23	*Agrobacterium tumefaciens* (95%)	*Agrobacterium tumefaciens* (90%)	*Sphingomonas sp.* strain DhA-33 (97%)

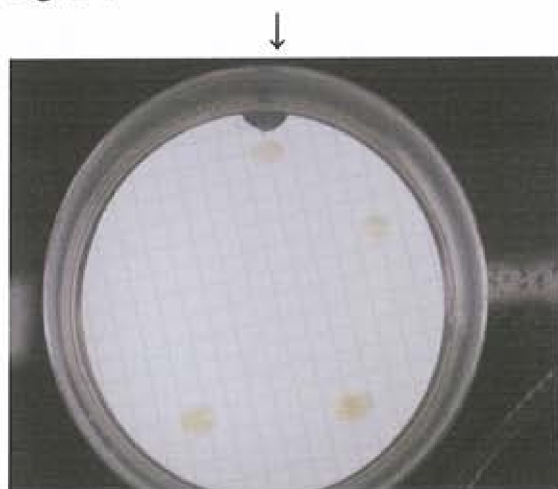
JM-CM 29	NO ID	NO ID	*Sphingomonas adhaesiva* (95.7%)
JM-CM 30	NO ID	NO ID	*Sphingomonas adhaesiva* (95.5%)
JM-CM 31	NO ID	NO ID	*Sphingomonas adhaesiva* (95%)
JM-HS 33	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HS 34	*Rhizobium rhizogenes* (80%)	*Rhizobium rhizogenes* (80%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HS 35	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HS 36	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HS 37	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HS 38	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HS 39	*Rhizobium rhizogenes* (80%)	*Rhizobium rhizogenes* (80%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HS 40	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HS 41	NO ID	NO ID	*Calobacter crescentus*(95%)
JM-HN 46	NO ID	NO ID	*Calobacter crescentus*(95%)
JM-HR 47	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HR 48	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HR 49	*Agrobacterium tumefaciens* (98%)	*Agrobacterium tumefaciens* (98%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HR 50	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HR 51	*Brevndimonas vesicularis* (84%)	*Brevndimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)

JM-HT 52	NO ID	NO ID	*Sphingomonas adhaesiva* (95%)
JM-HT 53	*Rhizobium rhizogenes* (80%)	*Rhizobium rhizogenes* (80%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HT 54	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HT 55	NO ID	NO ID	*Sphingomonas adhaesiva* (95%)
JM-HT 56	NO ID	NO ID	*Sphingomonas adhaesiva* (95%)
JM-HT 57	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HT 58	NO ID	NO ID	*Sphingomonas adhaesiva* (95%)
JM-HT 59	*Rhizobium rhizogenes* (80%)	*Rhizobium rhizogenes* (80%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HSB 60	*Brevundimonas vesicularis* (84%)	*Brevundimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HSB 61	*Agrobacterium tumefaciens* (98%)	*Agrobacterium tumefaciens* (98%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HSB 62	NO ID	NO ID	*Calobacter crescentus*(95%)
JM-HSB 63	*Brevundimonas vesicularis* (84%)	*Brevundimonas vesicularis*(84%)	*Sphingomonas aquatilis* (97%)
JM-HSB 64	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HSB 65	*Brevundimonas vesicularis* (84%)	*Brevundimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HSB 66	*Brevundimonas vesicularis* (84%)	*Brevundimonas vesicularis* (84%)	*Sphingomonas aquatilis* (97%)
JM-HSN 67	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HSN 68	NO ID	NO ID	*Sphingomonas aquatilis* (97%)
JM-HSN 69	NO ID	NO ID	*Calobacter crescentus* (95%)
JM-HSN 70	*Agrobacterium tumefaciens* (98%)	*Agrobacterium tumefaciens* (98%)	*Sphingomonas sp.* strain DhA-33 (97%)

JM-HSN 71	*Agrobacterium tumefaciens* (98%)	*Agrobacterium tumefaciens* (98%)	*Sphingomonas sp.* strain DhA-33 (97%)
JM-HSN 72	NO ID	NO ID	*Sphingomonas aquatilis* (97%)
JM-HSN 73	NO ID	NO ID	*Calobacter crescentus* (95%)

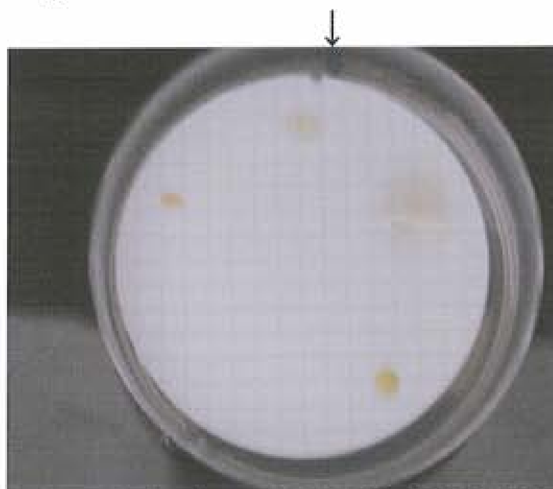
Legend of the Table: JM-AE (aeromonas medium), JM-CM (coliform isolates at 30 °C), JM-HS (HPC for Brand A), JM- HN (HPC for Brand B), JM-HR (HPC for Brand C), JM-HT (HPC for Brand D), JM-HSN (HPC for Brand F), JM-HSB (HPC for Brand E), JM-PS (*pseudomonas* medium), JM-CT (coliform isolates at 44.5°c), JM-EN(*Enterococcus medium).*

Figure 7



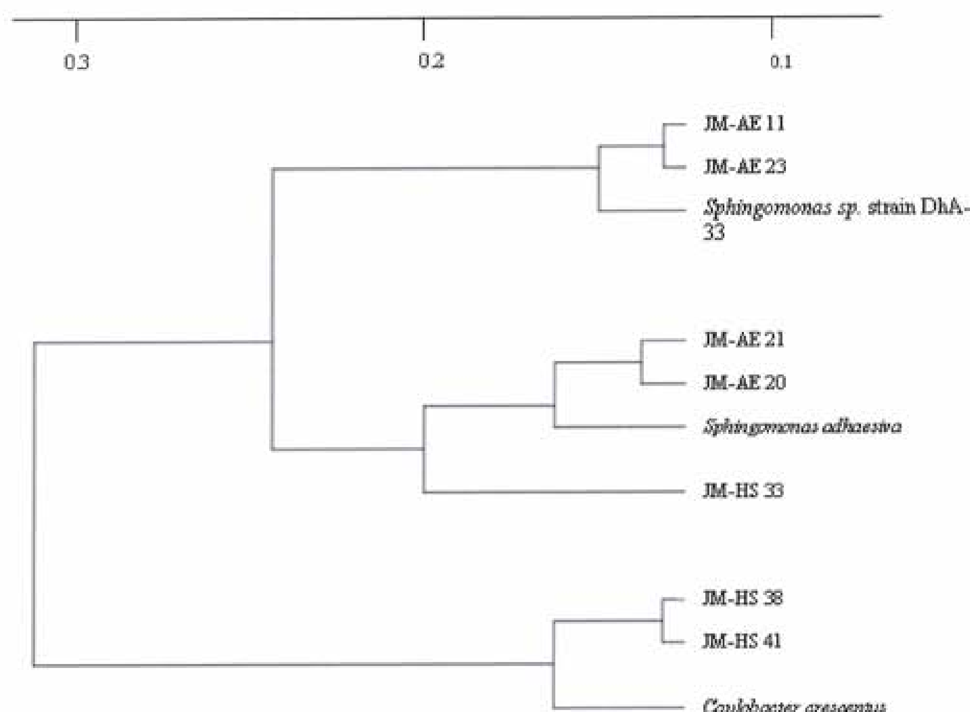
Going clockwise: JM-HS 38, JM-HS 41, JM-AE 11 and JM-AE 23.

Figure 8



Going clockwise: JM-HN 42, JM-HT 53, JM-AE 20 and JM- HS33

Figure 9
Phylogenetic tree relating sequence-based identification of organisms belonging to the α-*proteobacteria.* The algorithm used was the unweighted pair group method using averages (UPGMA). The tree was used to determine closest identification for isolates.



5.6.2 Isolated colonies belonging to the β-*proteobacteria.*

Figure 11 shows the morphology of *Stenotrophomonas maltophilia* as being white regular colonies

Table 25 : The Identification of organisms belonging to β-*proteobacteria* by using both Biolog and the 16S rDNA sequencing technique.

β-*proteobacteria*	Biolog Id #1	Biolog Id #2	16S rDNA sequencing Id.
JM-HN 42	*Stenotrophomonas maltophilia* (95%)	*Stenotrophomonas maltophilia* (95%)	*Stenotrophomonas maltophilia* (95%)
JM-HN 45	*Stenotrophomonas maltophilia* (95%)	*Stenotrophomonas maltophilia* (95%)	*Stenotrophomonas maltophilia* (95%)

5.6.3 Isolates identified belonging to the *γ-proteobacteria.*

Figure 12 shows the Pseudomonads isolates where *P.aeruginosa* has a pale green colour and *P.fluorescence* a dark bright green colour. The isolated aeromonads showed a white shiny colony morphology (Figure 10)

Table 26: The Identification of organisms belonging to *γ-proteobacteria* by using both Biolog and the 16S rDNA sequencing technique.

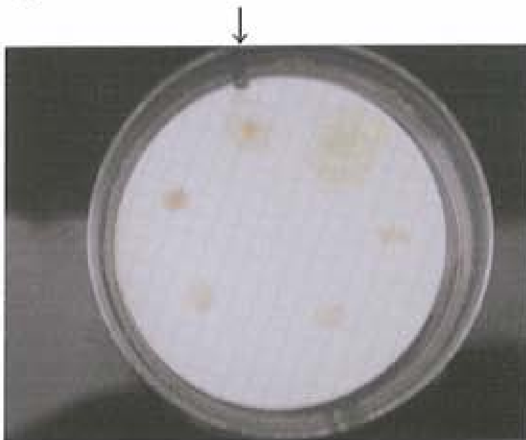
γ-proteobacteria	Biolog Id #1	Biolog Id #2	16S rDNA sequencing Id.
JM-PS 1	NO ID	NO ID	*Pseudomonas sp K1* (97%)
JM-PS 2	*Pseudomonas Sp.* (95%)	*Pseudomonas Sp.* (95%)	*Pseudomonas marginalis* (98%)
JM-PS 3	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (99%)	*Pseudomonas aeruginosa* (99%)
JM-PS 4	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (100%)	*Pseudomonas fluorescence* (98%)
JM-PS 5	*Pseudomonas mendocina* (95 %)	*Pseudomonas mendocina* (95%)	*Pseudomonas sp K1* (99%)
JM-PS 6	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (100%)	*Pseudomonas fluorescence* (97.6%)
JM-PS 7	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (100%)	*Pseudomonas fluorescence* (97.6%)
JM-PS 8	*Pseudomonas aeruginosa (95%)*	*Pseudomonas aeruginosa (100%)*	*Pseudomonas fluorescence* (97.6%)
JM-AE 14	*Aeromonas sp.* (95%)	*Aeromonas sp.* (95%)	*Aeromonas sp. CDC 715-84* (98%)
JM-AE 16	*Aeromonas sp.* (95%)	*Aeromonas sp.* (95%)	*Aeromonas sp. CDC 715-84* (97.5%)
JM-AE 17	*Aeromonas sp.* (95%)	*Aeromonas sp.* (95%)	*Aeromonas sp. CDC 715-84* (98%)
JM-AE 19	*Aeromonas sp.* (95%)	*Aeromonas sp.* (95%)	*Aeromonas sp. CDC 715-84* (98%)
JM-CM 27	*Pseudomonas Sp.* (95%)	*Pseudomonas Sp.*(95%)	*Pseudomonas marginalis*(98%)
JM-CM 28	*Pseudomonas aeruginosa*(99%)	*Pseudomonas aeruginosa*(95%)	*Pseudomonas aeruginosa*(99%)
JM-CT 32	NO ID	NO ID	*Pseudomonas aeruginosa* (99%)
JM-HN 43	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (99%)	*Pseudomonas aeruginosa* (99%)
JM-HN 44	*Pseudomonas aeruginosa* (100%)	*Pseudomonas aeruginosa* (99%)	*Pseudomonas aeruginosa* (99%)

5.6.4 Isolated Gram-positive organisms

Table 27 Table to show the Identification of Gram-positive organisms by both Biolog and 16S rDNA sequencing technique

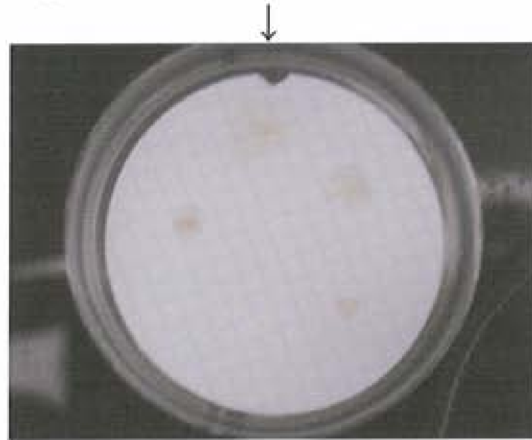
Gram +ve	Biolog Id #1	Biolog Id #2	16S rDNA sequencing Id.
JM-EN 24	NO ID	NO ID	*Micrococcus luteus* (98.5%)
JM-EN 25	NO ID	NO ID	*Micrococcus luteus* (98%)
JM-EN 26	NO ID	NO ID	*Micrococcus luteus* (98)%

Figure 10



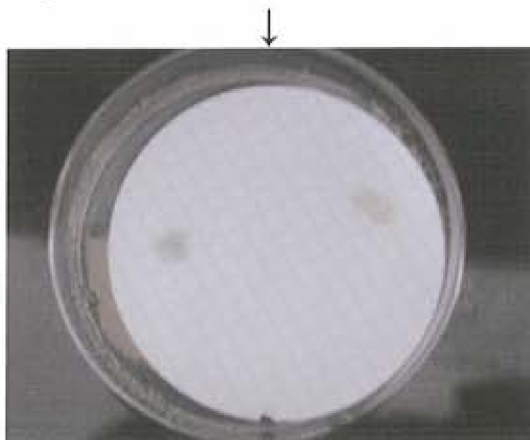
Going clockwise: JM-HT 53 , JM-PS 2, JM-AE 16, JM-AE 19, JM-EN 24 And JM-CM 29

Figure 11



Going clockwise: JM-HN 45, JM-PS 5, JM-AE 16, JM-EN 25 and JM-HN 42

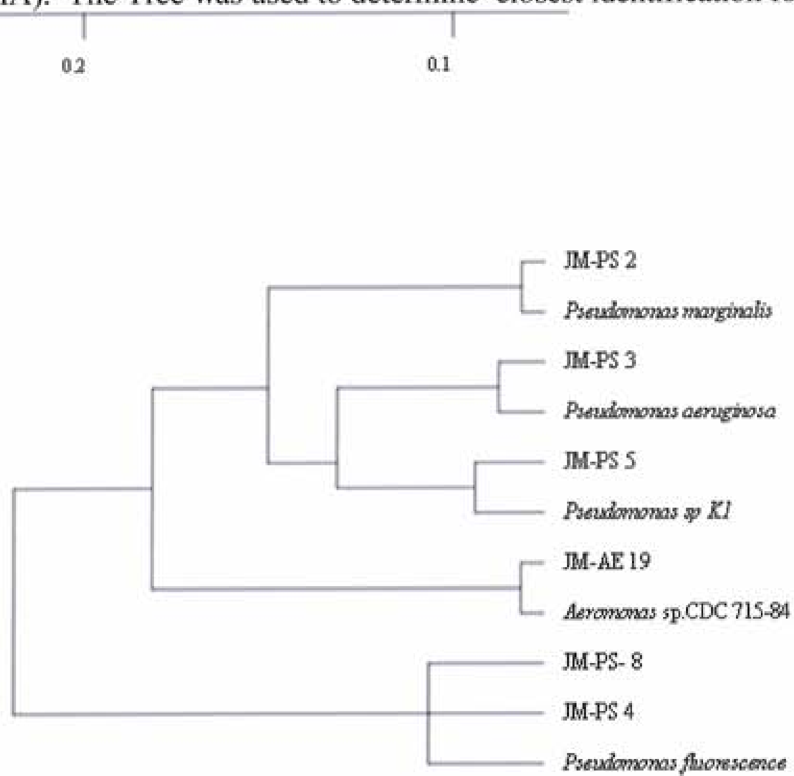
Figure 12



JM-PS 3 on the right and JM-PS 8 on the left

Figure 13

Phylogenetic tree relating sequence-based identification of bacteria belonging to γ-*proteobacteria*. The algorithm used was the unweighted pair group method using averages (UPGMA). The Tree was used to determine closest identification for isolates.



5.6.5 Biolog system versus 16S rDNA Sequencing

Figure 14: The percentage of samples which gave the same, different or no ID using the Biolog system as compared to the 16S rDNA sequencing technique.

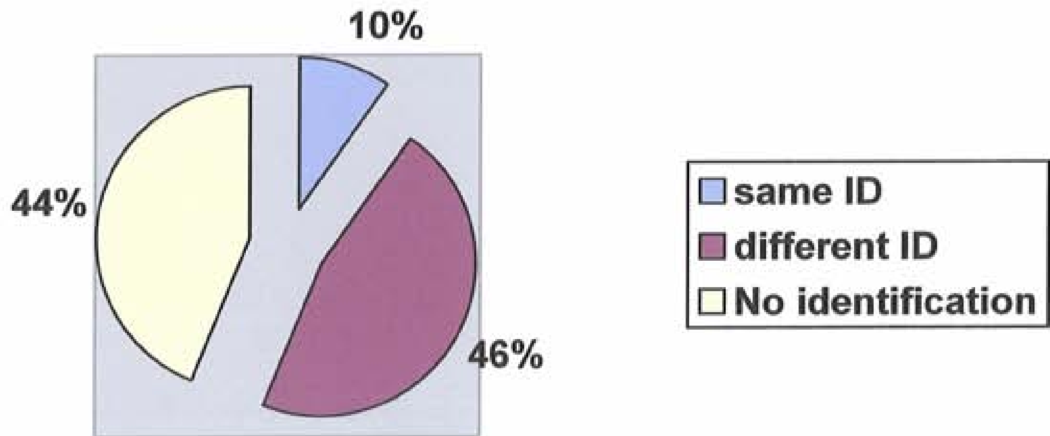


Figure 14 Shows that 7 colonies identified using the Biolog system had a matching ID with those obtained using the 16S rDNA sequencing technique, 90% of the samples gave a different ID and 44% showed no ID using the Biolog system.

5.7 16S rDNA sequencing based identification of isolates recovered in this study.

16S rDNA sequencing revealed that Sphingomonas sp. was recovered from the brands total in this study (Table 27,28,29,30). Pseudomonads however, were found in all brands except in Brand D and Brand F bottled water (Table 31,32). Interestingly *Aeromonas sp. CDC 715-84* was only detected in Brand C from 3 different batches and in Brand D from one batch taken during the summer (Table 29 and 31). Finally *Calobacter crescentus* was also a common isolate in all brands except for Brand C bottled water (Table 29).

Table 27 : Organism isolated and identified from Brand B bottled water

Brand B	Batch1	*Pseudomonas aeruginosa*	*Sphingomonas adhaesiva*	*Sphingomonas sp.* strain DhA-33
	Batch2	*Pseudomonas aeruginosa*	*Stenotrophomonas maltophilia*	-
	Batch3	*Pseudomonas fluorescence*	-	-
	Batch4	*Micrococcus luteus*	*Calobacter crescentus*	-

Table 28: Organism isolated and identified from Brand A bottled water

Brand A	Batch1	*Sphingomonas aquatilis*	*Micrococcus luteus*	-	-	-	-
	Batch2	*Sphingomonas sp.* strain DhA-33	*Sphingomonas aquatilis*	*Calobacter crescentus*	-	-	-
	Batch3	*Sphingomonas sp.* strain DhA-33	*Pseudomonas sp K1*	*Pseudomonas marginalis*	*Sphingomonas adhaesiva*	*Sphingomonas aquatilis*	*Calobacter crescentus*
	Batch4	*Sphingomonas sp.* strain DhA-33	*Sphingomonas aquatilis*	*Calobacter crescentus*	*Micrococcus luteus*	-	-

Table 29 Organism isolated and identified from Brand C bottled water

Brand C	Batch1	*Pseudomonas sp K1*	*Sphingomonas adhaesiva*	*Sphingomonas aquatilis*	-
	Batch2	*Aeromonas sp. CDC 715-84*	*Sphingomonas adhaesiva*	*Sphingomonas aquatilis*	*Pseudomonas fluorescence*
	Batch3	*Aeromonas sp. CDC 715-84*	*Sphingomonas sp.* strain DhA-33	*Sphingomonas aquatilis*	-
	Batch4	*Aeromonas sp. CDC 715-84*	*Sphingomonas aquatilis*	-	-

Table 30 Organism isolated and identified from Brand E bottled water

Brand E	Batch1	*Pseudomonas fluorescence*	*Sphingomonas sp.* strain DhA-33	*Sphingomonas adhaesiva*	*Sphingomonas aquatilis*
	Batch2	*Pseudomonas fluorescence*	*Calobacter crescentus*	*Sphingomonas aquatilis*	-
	Batch3	*Pseudomonas aeruginosa*	*Sphingomonas sp.* strain DhA-33	*Calobacter crescentus*	*Sphingomonas aquatilis*
	Batch4	*Sphingomonas aquatilis*	-	-	-

Table 31 Organism isolated and identified from Brand D bottled water

Brand D	Batch1	*Sphingomonas adhaesiva*	*Sphingomonas sp.* strain DhA-33	*Calobacter crescentus*
	Batch2	*Sphingomonas adhaesiva*	*Aeromonas sp. CDC 715-84*	-
	Batch3	*Sphingomonas adhaesiva*	*Calobacter crescentus*	-
	Batch4	*Sphingomonas adhaesiva*	*Sphingomonas sp.* strain DhA-33	-

Table 32 Organism isolated and identified from Brand F bottled water

Brand F	Batch1	*Calobacter crescentus*	-	-
	Batch2	*Sphingomonas aquatilis*	-	-
	Batch3	*Calobacter crescentus*	*Sphingomonas sp.* strain DhA-33	-
	Batch4	*Calobacter crescentus*	*Sphingomonas aquatilis*	*Sphingomonas sp.* strain DhA-33

6 Discussion

6.1 Enumeration of Bacteria

Thirteen presumptive colonies representing *P. aeruginosa* were recovered in this study. All thirteen isolated colonies were identified as *Pseudomonas* out of which 5 were *P. aeruginosa* (JM-PS 3, JM-CM 28, JM-CT 32, JM-HN 43 and JM-HN 44). Only one of the *P. aeruginosa* isolates was recovered on the pseudomonas agar (JM-PS 3), while the others were detected when testing for coliforms (lauryl tryptose agar) and total HPC (R2A agar). The medium is suitable to test for all pseudomonads, and not only *P. aeruginosa.* Only one *P. aeruginosa* isolate was recovered from a brand other than Brand B, and it was from Brand E bottled water (JM-CT 32) using lauryl tryptose agar at 44.5°C. Other colonies growing on pseudomonas agar were identified as *P. fluorescence, P. marginalis* and *Pseudomonas sp.* K1.

In this study 30.7% of recovered pseudomanads where confirmed as *P. aeruginosa* and this is in concordance with the findings of Hunter (1993) who reported that 29 % of *Pseudomonas sp.* strains isolated from bottled water were *P. aeruginosa.* In contrast, Mania et al. (1990) isolated *Pseudomonas sp.* from 83 % of batches of bottled water collected from retail outlets compared to only 30.5% of batches that were tested in this study. *P. aeruginosa* was also recovered on other types of media used including lauryl tryptose agar (JM-CM 28) and R2A agar from Brand B brand batches 1 (JM-HN 43) and 2 (JM-HN 44).

All the tested samples stood by the standard of 0 cfu/250 ml of *P. aeruginosa* except Brand B, where on Pseudomonas medium 55 cfu/250ml were isolated from batch 1. This finding when correlated with the HPC counts, where 20 *P. aeruginosa* cfu/ml were recovered (equivalent to 5000 colonies/250 ml), it far exceeds the set standard of 0 cfu/250ml. It is noteworthy that *P. aeruginosa* recovered from Brand B bottled water was from a winter batch (batch 1), but a seasonal impact was ruled out as none was recovered from the following winter tested batch.

Although the presence of A*eromonas spp.* in drinking water is considered as a potential health hazard, as these organisms produce a wide range of virulence factors (Ivanova, 2001), standards for A*eromonas spp.* in bottled waters have not been set

neither by the EPA or IBWA nor have recommendations been agreed upon. Similarly, Lebanese standards were also not set for *Aeromonas spp.* in drinking or bottled water (MOH, 1999). However, in Italy standards were set for natural mineral waters at their origin to be 10 colonies/100ml and after being bottled at 100 colonies/100ml these were maintained till the end of 1998 (Villari, 2003). The results of this study accordingly, were compared based on the Italian standards of 100 colonies/100ml. JM-AE 14 (9 colonies/ 100ml), JM-AE 16 (18 colonies/100ml), JM-AE 17 (8 colonies/ 100ml) and JM-AE 19 (46 cfu/ 100ml), were all aeromonads recovered during the course of this study, with the number in all cases being within the acceptable range. Of the 15 isolated colonies on the *Aeromonas* medium only 4 were confirmed to be *Aeromonas sp.* (26.7%), while all the remaining colonies growing on this medium were identified as *Sphingomonas sp.* This indicates that the medium is not selective enough and that other types of organisms can also be recovered, and thus results should be carefully analyzed. *Aeromonas* isolates were recovered from batch 2 (spring), batch 3 (summer) and batch 4 (winter) in Brand C bottled water. The counts increased respectively from 9 colonies/100ml to 18 colonies/100ml followed by a decrease to 8 colonies/100ml. *Aeromonas* was absent from batch 1 collected during the first winter of the study, while 8 colonies/100ml were detected in the second winter batch (batch 4), excluding the possibility of any seasonal correlation.

The presence of coliforms was monitored in all collected bottled drinking water samples. No coliforms were isolated using the lauryl tryptose agar, with the recovered presumptive coliforms being either pseudomonads or sphingomonads. In the third batch of Brand A bottled water 2 colonies were recovered and identified as *P. marginalis* (JM-CM 27), while the one recovered from Brand B batch 1 was identified as *P. aeruginosa* (JM-CM 28), and was additionally isolated using pseudomonas agar. Presumptive coliforms were also detected in the other tested batches and the identification of those colonies revealed that those from Brand C batch 1 (JM-CM 29), Brand D batch 3 (JM-CM 30) and Brand E batch 1 (JM-CM 31) were not coliforms but rather *Shpingomonas adhaesiva.*

Faecal coliforms were not detected in any of the tested bottled drinking water samples. The United States Environmental Protection Agency (EPA) indicated that the

presence of total coliforms is a possible health concern, while the detection of faecal coliforms specifically *E. coli*, is considered as a serious health hazard because of their association with sewage and/or animal wastes. The fluctuation observed in the total and faecal coliform counts was not significant as the calculated averages when rounded would be close to 0 cfu/100ml. This was additionally confirmed, upon the identification of the recovered presumptive colonies, which were not coliforms but rather *P. aeruginosa* (JM-CT 32). Thus bottled drinking water samples tested were within the acceptable set standards, but the isolation of *P. aeruginosa* in Brand B and Brand E should not be dismissed as these were recovered on lauryl tryptose agar and considered as presumptive coliforms.

Only three presumptive enterococci were recovered on the enterococcosel agar, JM-EN 24 and JM-EN 25 from Brand A batch 1 and 4 respectively and JM-EN 26 from Brand B batch 4. However, these isolates were all identified as *Micrococcus luteus.* Enterococci are important as a leading cause of nosocomial infections, and having a significant role in the dissemination and persistence of antimicrobial resistance (Jackson, 2004). Therefore their presence in drinking and bottled water is unacceptable.

Determination of the Heterotrophic plate count (HPC) bacteria in bottled water can be helpful in assessing water quality, which is useful in determining changes in water quality both during storage and distribution (Carter et al., 2000). In epidemiological studies conducted by Payment et al. (1991), the potential negative impact on human health from the consumption of treated water containing high HPC levels of bacteria was not resolved.

Brand A, Brand B and Brand F all had a mean HPC (in all four batches) less than 500 cfu/ml, while the number was 677 cfu/ml in Brand E bottled water. However, Brand C and Brand D had mean HPC counts of 4022 cfu/ml and 4923 cfu/ml, respectively. Although there has been no association between HPC bacteria and human illness, the IBWA and EPA have recommended that the HPC should not exceed 500 cfu/ ml at 28 °C when incubated for 5-7 days. The results of this study showed that the HPC bacteria recovered from Brand D and Brand C far exceeded the acceptable values, whether according to international or Lebanese standards. The HPC for Brand E bottled drinking

water although exceeded the international recommendations, but it was within the accepted Lebanese standards.

6.1.1 HPC fluctuations between batches in each brand

BRAND A

The difference between the batches is shown in Table 18. All tested batches had relatively comparable values, except batch 2. Batches 1 and 4 had values falling within one standard deviation of the mean, compared to batches 2 and 3, which fell within two standard deviations of the mean. Both winter batches had comparable values, the spring showed the lowest value and the summer batch had the highest. Identification of the recovered isolates revealed that *Caulobacter crescentus, Sp. aquatilis, Sphingomonas sp.* strain DhA-33 and *Sp. aquatilis* were present in almost all tested batches. Accordingly, we can assume that the Brand A brand was bottled from a fixed source at least during the course of this study.

BRAND B

The differences between the numbers of colonies in the different tested batches are shown in Table 19. A steady increase was detected in batches 1 and 2; bacteria were not detected in batch 3 and only 18 colonies/ml were recovered from batch 4. The difference observed in the number of colonies between batch 1 (200 colonies/ml) and batch 2 (442 colonies/ml), could be attributed either to a modification being introduced in the water processing procedure, or a change in the water source or probably both combined. To examine the possibility of a change in the water source, the types of organisms recovered were analyzed (Table 27), and the results revealed the lack of common isolates in the tested batches. Accordingly, although the HPC counts didn't exceed the standards set for bottled water, but it certainly did indicate a change in the water source.

BRAND C

A drastic difference was detected between the HPC value in batch 1 and that of batch 2 (Table 20), while the mean count number for batch 3 dropped to 4022 colonies/ml and that of batch 4 to 46 colonies/ml. *Aeromonas sp.* CDC 715-84 was isolated from 75% of batches tested, while *Sp. aquatilis* was recovered from all batches undertaken in this

study, indicating that the water source was the same and the observed fluctuations in HPC values could be attributed to the water processing.

BRAND D

A steady linear increase in HPC counts from one batch to the other was reported for Brand D bottled water (Table 21). The HPC values obtained for Brand D during the course of this study showed that there was a linear increase in the HPC count throughout the year. No assumption on the affect of the season on the HPC can be made since the second winter batch had a higher value than the first winter batch. The water source used in the case of Brand D was most probably fixed, an observation supported by the recovery of *Sp. adhaesiva* from all tested batches.

BRAND E

In the first three batches the HPC values in Brand E water were relatively comparable, while a significant change was detected in batch 4 (Table 22). As *Sp. aquatilis* was a common isolate in all tested batches, it can be concluded that the source was the same and the change was introduced during the processing of water before bottling.

BRAND F

HPC values for batches 1, 3 and 4 were relatively comparable with a sudden increase being observed in batch 2. However, the fluctuations observed all fell within the set standards including the unusual increase observed in batch 2. *C. crescentus* was the common isolate recovered from all tested batches, except for batch 2. The unusual increase in batch 2 was again attributed to processing rather than to the source taking into consideration that the increase was mainly due to *Sp. aquatilis.*.

6.2 IDENTIFICATION OF ISOLATED BACTERIAL

6.2.1 BIOLOG IDENTIFICATION:

The traditional methods used for bacterial identification rely on phenotypic methods. Among which the most common and widely used commercial identification systems are the API (bioMerieux, France) and the Biolog (Biolog inc., USA). A problem facing the identification of bacteria using phenotypic methods is that some strains express phenotypic variability, which would present atypical characteristics for successful identification (Drancourt *et al.,* 2000). Tokajian and Hashwa (2004) found that the 51% of samples gave similar identities using the Biolog and API systems.

The identification of organisms using the Biolog and API systems in this study revealed that with 46% of the samples different results were obtained (Tables 24-27), while 44% were not identified using the Biolog system (Figure 13). Only 10% of the samples had a matching ID when identified using the Biolog and 16S rDNA sequencing. Organisms with matching IDs were *Pseudomonas sp.* and *Stenotrophomonas maltophilia. C. crescentus* and *Sp. adhaesiva* however, were not identified using the Biolog system, which is in harmony with the fact that most of the available phenotypic methods were designed to work with clinical isolates.

6.2.2 16S rDNA Identification

Recent advances in DNA sequencing technology greatly enhanced the identification of bacterial isolates and facilitated the shift from the use of traditional methods to molecular techniques (Kolber and Persing, 1999). The 16S rDNA sequencing based identification of bacteria has emerged as an attractive alternative to traditional methods (Boye et al.,1999). The 16S rather than the 23S part of the rDNA are more widely used because the 16S rDNA is more manageable due to its smaller size even though the 23S allows greater resolution (Brock, 1988). The widespread availability of published 16S rDNA sequences and availability of a wide range of databases renders the use of the 16S rDNA highly suitable for definitive bacterial identification (Kolbert and persing, 1999). 16S rDNA provides phylogenetically useful information (Brock, 1988; Kolbert and persing, 1999). In contrast, the Biolog system gave several ambiguous results and 44% of samples were not identified at all.

Identifications of all the samples collected were obtained using the EMBL with similarity values above 95%. Identified samples were separated into α, β and γ subgroups of *Proteobacteria.* Phylogentic trees were then drawn for a sample of the isolates since most were the same although being isolated from different batches and on different media. The 16S rDNA identification resolved the ambiguity observed with the Biolog identification system, where some of recovered organisms in this study (JM-PS 4, JM-PS 6, JM-PS 7 and JM-PS 8) were identified as *P. aeruginosa* at 100% similarity using the Biolog, while the 16S rDNA sequencing revealed that they were actually *P. fluorescence*.

Sphingomonas spp. was isolated from 83.3% of batches tested in this study. Tokajian (2002) found that 61% of bacterial population in water storage tanks belonged to α-proteobacteria of which 95% was identified to be *Sphingomonas* spp. and that their presence in water distribution networks (tap water) is much more common than reported and their incidence is high. The highest count of *Sphingomonas* spp. isolated was 9617 colonies/ml in batch 2 of brand C and 5400 colonies/ml in batch 3 of brand D.

Aeromonas spp. were isolated by Tokajian(2002) in tap water from water distribution systems in Lebanon when the level of chlorination was low. In Brand D *Aeromonas* spp. was isolated from 75% of the batches with an average count of 11 colonies/250ml.

Therefore from the bacterial content of bottled water there are times that bottled water is no better than tap water since the incidence of bacteria predominant in tap water are found in bottled water in high numbers such as is the case in brand C and D.

7 CONCLUSIONS:

Since the Biolog system proved to be unreliable conclusions will be drawn based on the 16S rDNA sequencing Identification

- The water quality of bottled water tested
    - The overall microbial quality of Brand A and F are the most acceptable and within all standards
    - The microbial quality of Brand B and E were acceptable in general with the occasional presence of *Pseudomonas aeruginosa* in both brands.
    - *Aeromonas sp.* CDC 715-84 was isolated from 75% of the tested Brand C bottles, with numbers that were within the acceptable range. The HPC count was very high and did not adhere to international or Lebanese standards in 50% of the tested batches.
    - *Aeromonas sp.* CDC 715-84 was isolated from Brand D tested bottles, but again the numbers were within the acceptable range. The high HPC values detected were not within neither international nor Lebanese standards with 100% of the batches.
- Brand B was not bottled from one fixed source. This was concluded from the HPC values and the variation observed between the batches with respect to the type of detected organisms. The results obtained also revealed that batch 4 of the Nestle bottled water was taken from the same source as that of Brand A water, as indicated by the number of *Caulobacter crescentus* and *Micrococcus luteus* present in both brands

- According to the results in this study a bacterial fingerprint can be assigned for the tested brands:
    - Brand A: *Sphingomonas aquatilis* in all batches, *Caulobacter crescentus* and *Sphingomonas sp.* strain DhA-33 in 75% of the batches
    - Brand C: *Sphingomonas aquatilis* in all batches while *Aeromonas sp.* CDC 715-84 in 75% of the batches
    - Brand D: *Sphingomonas adhaesiva* in all batches
    - Brand E: *Sphingomonas aquatilis* in all batches
    - Brand F: *Caulobacter crescentus* in 75% of the batches
- Seasonal variation did not have any significant effect on water quality.
- Results obtained using the Biolog system for the identification of environmental samples should be carefully interpreted.
- The quality of bottled drinking water in Lebanon should be properly monitored.

Reccomendations for future work

- All brands available on the market should be analaysed
- Number of batches should be increased
- Compare water quality of batches to those of tap water from the same season

8- References

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9- Appendix

Table to show a summary of all quantitative results in this study.

	Number Of Presumptive Isolates						
		Pseudomonads	*Aeromonads*	*Enterococci*	**coliforms**	**Faecal coliforms**	**HPC/ml**
Brand A	Batch 1	0	0	1	0	0	382
	Batch 2	0	0	0	0	0	7
	Batch 3	15	43	0	2	0	533
	Batch 4	0	0	4	0	0	179
Brand B	Batch 1	22	111	0	1	0	200
	Batch 2	0	0	0	0	0	442
	Batch 3	1	0	0	0	0	0
	Batch 4	0	0	3	0	0	18
Brand c	Batch 1	82	721	0	1	0	5496
	Batch 2	7	100	0	0	0	9617
	Batch 3	0	18	0	0	0	520
	Batch 4	0	8	0	0	0	456
Brand D	Batch 1	0	60	0	0	0	3899
	Batch 2	0	769	0	0	0	4530
	Batch 3	0	361	0	2	0	5413
	Batch 4	0	972	0	0	0	5849
Brand E	Batch 1	42	0	0	1	0	581
	Batch 2	20	0	0	0	0	634
	Batch 3	0	0	0	0	4	623
	Batch 4	0	1	0	0	0	870
Brand F	Batch 1	0	0	0	0	0	29
	Batch 2	0	0	0	0	0	517
	Batch 3	0	0	0	0	0	10
	Batch 4	0	0	0	0	0	18