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Microbiological safety of natural mineral water

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Abstract

Natural mineral water originates from groundwater, an oligotrophic ecosystem where the level of organic matter is low and of a very limited bioavailability. The bacterial populations that evolve in these ecosystems are heterotrophic and in starvation–survival state resulting from an insufficient amount of nutrients; for this reason they enter a viable but non-culturable state. After bottling, the number of viable counts increases rapidly, attaining 10^4–10^5 colony-forming units ml^{-1} within 3–7 days. These bacterial communities, identified by culture or with specific probes, are primarily aerobic, saprophytic, Gram-negative rods. Groundwater sources for natural mineral waters are selected such that they are not vulnerable to fecal contamination. Ecological data, especially the diversity and physiological properties of bacterial communities, are essential together with epidemiological studies in order to perform a risk analysis for natural mineral waters. On a continuing basis, the management of microbial risks has to rely on assessment of the heterotrophic plate count and, more specially, on detection of marker organisms, i.e. the classic fecal contamination indicators that have to be absent, and vulnerability indicators for which the occurrence should be as low as possible. It is also recommended to search regularly, but not routinely, for viral and protozoan pathogens.

Keywords: Natural mineral water; Risk assessment; Indicator of fecal contamination; Starvation–survival; Waterborne disease; Viable but non-culturable bacterium

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1. Introduction

The epidemiological history of waterborne diseases after the 1970s is marked by the emergence of enteric viruses and protozoans. Many of them are resistant to conventional chlorination and have thus been involved in waterborne outbreaks. Other new pathogens include species of environmental bacteria that are capable of growing in water supply networks, such as *Legionella* spp., *Aeromonas* spp., *Mycobacterium* spp. and *Pseudomonas aeruginosa*. These new epidemiological data have questioned the ability of conventional treatment to provide a safe supply of water and the use of marker organisms such as coliforms to monitor the efficiency of water treatment.

Natural mineral water is defined as microbiologically wholesome water, since based on experience it is protected from all risk of pollution through the intrinsic physical characteristics of the aquifer material. It therefore appears that mineral water can be clearly distinguished from drinking water from both an ecological and epidemiological point of view. Therefore the microbial risk assessment, needed for the development of better regulations, should also be distinct.

2. Groundwater habitat

Before the 1970s, the study of life in groundwater habitats was relatively limited. In the 1970s, however, it became increasingly obvious that certain waste disposal practices were contaminating subsurface environments with effects on groundwater quality. This led to the current interest in the study of these environments. There has also been an increasing interest in demonstrating that various shallow and deep environments contain substantial numbers of viable microorganisms and in using the ability of these microorganisms to degrade potential pollutants, i.e. in bioremediation. Subsurface microbiological research to study microbial community structure, microbial activities and the geochemical properties of groundwater environments has progressed with the development of aseptic sampling techniques [1,2].

In a hydrogeological sense, groundwater refers to water that is easily extractable from saturated, highly permeable strata known as aquifers. For saturated environments, a rigorous distinction between local, intermediate, and regional flow systems, related to the topography of recharge and discharge areas, has been long recognized by hydrologists [2]. One can thus define several underground aquifers that serve as source of potable water in the world, including shallow aquifers, intermediate and deep aquifers [3]. Shallow aquifers are characterized by active flow (meters per day) strongly influenced by local precipitation events. Intermediate aquifers within 300 m of the surface soil are separated from shallow aquifers by confining layers; they have much slower flow rates, of the order of meters per year. Deep aquifers are also confined, but more than 300 m below the subsurface soil and they are characterized by extremely slow flow rates (meters per century). Natural mineral waters originate mainly from intermediate aquifers characterized by a well defined and protected catchment area and a long underground transit time. In this kind of aquifer, often with fine-textured soils, constancy of flow rate, of chemical composition and temperature stability, are at all times and under all conditions ensured.

2.1. Biological component

The colonization of subsurface habitats has generated interest and speculation among geologists and microbiologists. Comparisons of microbial communities within vertical profiles extending from surface soil to subsurface sediments have shown that the bacteria found in different geological strata can be morphologically and physiologically distinct, but they do not reveal the developmental history of the subsurface microflora. Several hypotheses have been discussed by Madsen and Ghiorse [1]. The most plausible is that the stratified distribution of subsurface microorganisms could have been caused by vertical and horizontal colonization patterns by waterborne soil microorganisms via hydrological flow. Most of the experimental evidence to explain the origin of subsurface microorganisms addresses their transport from surface environments.

To deal with bacterial abundance and distribution in subsurface environments it is necessary to use specific methods for sampling sediments. The major difficulty in studying these environments is their relative inaccessibility, making them very difficult to sample. In most cases, the groundwater samples are collected through pumping from the aquifer. Unfortunately, there are several potential

problems with such a method. The first is that the drilling process can contaminate the environment under study, and it may be difficult to know whether the bacteria recovered are autochthonous or introduced by drilling. A second problem is the fact that a new environment is created by drilling. The alteration of the physical conditions by drilling may significantly affect microbial processes in the vicinity of the well. A third problem with sampling water from wells for microbiological analysis is that most microorganisms in the subsurface tend to be particle-bound. Thus, the species and the types of free-living microorganisms may be significantly different from those attached to particle surfaces. The question of attached or unattached microbial community was raised by microbiologists during early inquiries into the nature and the distribution of the subsurface microflora. It is generally accepted that the majority of bacteria in most ecosystems are attached to surfaces and are not suspended in the aqueous phase, as are planktonic bacteria. Major distinctions between sediment-bound and unattached bacteria were documented by Madsen and Ghiorse [1]. The available information suggests that an unattached groundwater community may not be a valid surrogate for the indigenous aquifer microflora which largely consists of attached bacteria. Unattached and attached groundwater communities appear to be different, though they probably represent overlapping components of a dynamic community. It can be concluded that there is likely to be a constant exchange of organisms between the attached and unattached communities.

The most commonly used methods to study the microbial ecology of subsurface environments include direct observation, culture techniques, nucleic acid-based methods, biochemical marker techniques, activity measurements in microcosms, ecological modelling and groundwater geochemistry [2,4,5]. The difference in scale between the microorganisms and their habitat is a basic conflict in these studies. In selecting a suitable scale for observing or culturing microorganisms, poor and relative information regarding the overall aquifer system may result. Most of these techniques make observations at a small scale (1–1000 g of aquifer material) or microscale (less than 1 g). Indeed the only large-scale observation that can be made concerns groundwater geochemistry.

Madsen and Ghiorse [1] have presented a generalized model for the relationship between geological stratigraphy and microbiological parameters. In going across soil horizons, the bacterial abundance decreases in direct proportion with nutrient levels. Below the soil horizons, microbial abundance increases substantially at the water table and, just above, in the capillary fringe zone. It can be speculated that the interface zones between the unsaturated and saturated zones may be the sites of oxygen transport and nutrients recharge, especially in shallow unconfined aquifers. Thus, depth per se appears not to govern bacterial abundance and activity in the saturated zone. Rather, it could be related to hydrological, physical and geochemical properties of each stratum.

Within a given environment, the collective result of all microbial processes, most of which are oxidation–reduction reactions, is viewed as a microbial food chain. The process itself is an important indicator in determining the nature of the microbial community in a particular habitat. The food chain in aquifers is primarily heterotrophic, reliant either upon an influx of dissolved organic carbon (DOC) along a given hydrological flow path or organic compounds of sedimentary origin that subsequently may become degraded. Thus the microbiological investigation of groundwater indicates that heterotrophic bacteria (prokaryotes) are the dominant microorganisms present while eukaryotes might be absent altogether or only present in low numbers. In subsurface environments, where photosynthesis does not occur, cyanobacteria and algae will not be found, unless the geological stratum is hydrologically connected to surface water. These organisms may be present in the form of cysts or other resistant states of development, their presence in groundwater is most likely to be the result of contamination through sampling. A modest population density of cyst-forming protozoa, among which flagellates and amoebae appear to be the principal types, may be expected to occur in pristine aquifer sediments. The population of fungi appears to be low, but not necessarily absent. The heterotrophic bacteria are capable of growing on simple organic compounds found in culture media such as R2A agar [6], largely used for monitoring drinking water. Because groundwater ecosystems are oligotrophic, these bacteria are often termed oligotrophic; in fact many of them are facultative oligotrophic, often capable of adapting to both low and high concentrations of organic substrates.

3. Starvation–survival lifestyle

Due to the long time the indigenous bacteria have had to degrade the organic matter originally present, subsurface environments contain little organic matter. Furthermore, when percolating through the porous media, water containing organic matter encounters attached bacteria which remove most of this organic matter. Thus, subsurface systems are oligotrophic and the intermediate aquifer flow systems are among the most oligotrophic microbial environments that have ever been described [7]. The average concentration of DOC for various types of consolidated rock aquifers ranges from 0.1 to 0.7 mg l^{-1}. Chemical analysis of the organic carbon in any environmental sample certainly does not determine what portion is available for use by the autochthonous bacteria. Most of the organic matter in subsurface environments, other than the readily labile compounds such as free amino acids, free carbohydrates, and free fatty acids, is humic, polymeric material high in molecular mass and refractory, i.e. resis-

tant to breakdown. Humic substances have extremely complex structures, and can be divided into three major fractions defined in terms of their solubility in water: humic acid, fulvic acid and humin. In the subsurface environments, it can be supposed that the unavailable humic and fulvic acids make up more than 50% of the total organic carbon.

Microbes have evolved longer than any other living organism, so in all probability, the non-spore-forming heterotrophic bacteria must have developed mechanisms to survive long periods when no energy or nutrients were available. Thus, the concept of starvation–survival is fundamental for the evolutionary point of view. In order to provide a pragmatic approach to this concept, a definition has been provided by Morita [8]: 'starvation–survival is a physiological state resulting from an insufficient amount of nutrients, especially energy, to permit growth (cell size increase) and/or reproduction'. There are various degrees of starvation, starting with cells that just utilized the last amount of nutrients for growth, to cells that have been deprived of nutrients for long periods of time.

To confront nutrient limitation, bacteria may develop defence mechanisms to enhance their ability to survive periods of starvation. Some differentiating bacteria respond to starvation by marked alteration in their ultrastructure, producing spores or cysts. Spores are essentially dormant, waiting out lean periods to germinate as nutrients become available. Non-differentiating bacteria respond more by an alteration of their physiology rather than developing resistant structural modifications [9,10]. (i) According to Morita [7] when bacteria are grown under conditions of nutrient excess, they accumulate reserve carbon polymers, such as glycogen and poly-β-hydroxybutyric acid RNA, protein, etc. The utilization of these cellular constituents during starvation is manifested by a basal level of endogenous metabolism which can be measured by oxygen consumption, CO_2 production or other tests. Thus bacteria that are more able to utilize endogenous macromolecules at a slow rate appear to have a specific advantage over other bacteria. (ii) Bacteria respond to specific nutrient limitation by two mechanisms: first, they produce transport systems with increased affinities for the nutrient most easily exploited; secondly, they express transport and metabolic systems for alternative nutrients. Thus, these bacteria may be able to escape starvation by more efficient scavenging of a preferred nutrient or by using another, relatively more abundant, source. The physiological and genetic basis of enhanced assimilation capacity for nitrogen, phosphorus, iron and carbon has been largely developed in many reviews [7,10–12]. These studies have revealed a large number of proteins that are induced by carbon starvation and required cyclic AMP for this induction. These proteins, termed the Cst (carbon starvation) proteins [13], are believed to be primarily concerned with escape from carbon starvation. (iii) Evidence has been accumulating for years that bacteria subjected to nutrient starvation become more resistant to various environmental stresses. It is clear that the stress responses discussed above, involving enhanced scavenging capacity, are insufficient to ensure survival. It has been shown that, upon exposure to nutrient limitation, bacteria synthesized new proteins that increased their resistance to a number of stresses including shifts in temperature, acid, oxidative and osmotic shock, uptake of antibiotics. This resistance failed to develop if synthesis of starvation proteins was prevented, and increased the longer the culture was allowed to synthesize the starvation proteins [13]. These additional proteins are often referred to as stress proteins.

Four patterns of starvation–survival have been described [7]. The most frequently pattern noted which might be representative for most environmental bacteria shows an initial increase in cell number due to fragmentation (reductive division) followed by a decline. The starvation pattern with time occurs in three stages, as that has been demonstrated by Moyer and Morita [14] in the marine bacteria Ant-300. During the first stage lasting 14 days, large fluctuations in plate counts were noted. In the second stage (14–70 days) the colony count decreased by 99.7%. The third stage was marked by a stabilization of viable cells (0.3% of the total count numbers). Cells in this stage of metabolic arrest have been termed 'shut down' cells by Dow et al. [15].

Cells resulting from nutrient starvation are mainly microcells defined as being 0.3 μm or smaller (also termed ultramicrocells or ultramicrobacteria). As a consequence of forming ultramicrocells, the surface/volume ratio becomes larger and leads to sequester nutrients more efficiently in low-nutrient environments. This is listed as a paramount characteristic of the model oligotrophs [7]. The presence of ultramicrocells in natural mineral water, capable of passing through a 0.2-μm filter, has been demonstrated [16].

3.1. The viable but non-culturable (VBNC) state

Under certain conditions of metabolic stress such as starvation, bacterial cells may enter a VBNC state. It has been realized for some time that plate counts can dramatically underestimate the total number of bacteria, determined by acridine orange (AO), present in samples taken from the natural environment. In the late 1970s, several non-cultural methods were developed for determining cell viability, which demonstrated that many of these unculturable cells are indeed viable and able to actively metabolize [17]. A bacterium in this VBNC state is defined by Oliver [17] as 'a cell which can be demonstrated to be metabolically active, while being incapable of undergoing the sustained cellular division required for growth in or on a medium normally supporting growth of that cell'. The relationship between viable cell counts (plate counts), total cell counts (AO direct counts or AODC) and respiring cell counts (INT) during starvation of a *Pseudomonas* spp. has

been studied by Kurath and Morita [18]. The difference observed between viable and INT counts suggests the existence within the starving population of a subpopulation of non-viable cells but having INT activity that is about 10-fold more numerous than the viable cells. These respiring bacteria that did not have the ability to form colony-forming units (CFU) on agar media might represent the predominant bacterial inhabitants of subsurface habitats. Cells entering the VBNC state generally show a reduction in size as has been noted for cells undergoing starvation.

It has been speculated that the VBNC state represents an additional response to starvation displayed by bacteria for survival [19] and many bacteria species, including pathogens, have been reported to enter this state under laboratory or field conditions [17]. This state could have important consequences with regard to ecology, epidemiology or pathogenesis, since potentially pathogenic VBNC cells could persist in the environment and regain growth capability and infectivity much later than normal for vegetative cells.

The relationship between the starvation response and the VBNC response is complex, but it has been suggested that the VBNC state may be distinct from the starvation response for several motives [7]. A large number of environmental factors other than starvation, such as temperature, pH, salinity and osmotic pressure, may be involved in the induction of the VBNC state. Cross-protection has not been demonstrated for bacteria entering the non-culturable state. It is important to note that starved bacteria, after variable periods of time, respond rapidly to nutrients, while VBNC cells cannot grow on conventional bacteriological culture plates. The existence of a VBNC state, in response to natural environmental stress, has been observed more often than not with Gram-negative bacteria representing members of the Enterobacteriaceae, Vibrionaceae including *Aeromonas* and some genera such as *Campylobacter*, *Helicobacter*, and *Legionella*. However, little is known about the VBNC state in most representative bacteria living in groundwater habitats.

Recent data concerning the physiology and biochemistry of starved *Escherichia coli* cells favor the model that starvation-induced loss of culturability is the result of gradual deterioration rather than a programmed and adaptive phenomenon [20].

4. Bottle habitat

Microbiological analysis of natural mineral water at source has always revealed the presence of some bacteria that are capable of growth and can form colonies on appropriate culture media. After bottling, the number of viable counts increases rapidly, attaining 10^4–10^5 CFU ml^{-1} within 3–7 days [5,21]. During the following weeks, the bacterial counts decrease slowly or remain fairly constant. At the end of 2 years storage, colony counts are still about 10^3 CFU ml^{-1} [22]. The heterotrophic bacteria selected during the times are psychrotrophic, because they can grow at temperatures as low as 5°C, and their maximum growth temperature is about 35°C. Furthermore, they do not have growth factor requirements such as vitamins, amino acids or nucleotides and are, therefore, prototrophic, in contrast to auxotrophic bacteria which require many of these growth factors.

The rapid multiplication of heterotrophic bacteria in flasks containing natural mineral water has been documented by many investigators [7]. However, possible explanation of this phenomenon is under much debate.

4.1. The bottle effect

Placing samples into containers terminates the exchange between cells, nutrients, and metabolites with the in situ surrounding environment. Compressed air is used at virtually all stages of the water-bottling process. The microbiological quality of the process air must be of a very high standard. On the other hand, the complexed organic matter present in low concentration can be dramatically modified through bottling, under the influence of increasing temperature and oxygenation. A high increase in cell numbers due to the bottle effect was reported for the first time by Fred et al. [23]. ZoBell and Anderson [24] described the bottle effect (originally named the volume effect) observing that both the number of bacteria and their metabolic activity were proportional to the surface area/volume ratio of the flask in which the seawater was stored. The greater the surface area in relation to the volume of water, the more rapidly growth of bacteria takes place, hence small containers provide considerably more surface area [7,22]. The explanation for this is that nutrients present in low concentration are adsorbed and concentrated onto the surface and, thus, can be more available for the bacteria. The similar increase in bacterial numbers occurs when underground or surface waters are placed in a container [25]. Flask surface adsorption of organic matter is the basis for the adhesion of bacteria to solid surfaces as demonstrated in both the aquatic environment and in the laboratory, and because of the increased concentration, the nutrients are more available [7]. It is also possible that many of the more labile compounds, unavailable at the subsurface environments being complexed to lignins, phenolics, or adsorbed to clay minerals, become biodegradable through sampling by interaction at the surfaces. Since a volume effect has been reported, the major portion of the microbial activity should be linked to the attached bacteria. To date, little experimental evidence has been presented to demonstrate an attachment of bacteria with the inner surfaces of the bottles of mineral water. Bischofberger et al. [7] reported no visible colonization with polyvinyl chloride (PVC) mineral water bottles, using scanning electron microscopy. Low levels of adhesion have been shown by Jones et al. [26]. Viable counts on the surfaces (polyethyl-

eneterephthalate bottles and high density polyethylene caps) ranged from 11 to 632 CFU cm^{-2} representing only 0.03–1.79% of the total viable counts in the 1.5-l bottles, depending on the brand examined. In contrast, within the studies of Jayasekara et al. [27] who reported considerable variation between bottles for a given producer of water, up to 83% of the total microbial population within a bottle was found to be adhered to the bottle surface, representing a population of about 10^6–10^7 CFU scattered over the surface. However, the counts of the attached bacteria were insufficient to constitute a real biofilm as representing an interdependent community-based existence [28]. The studies cited above [26,27] are not all directly comparable because there are differences in sampling and methods used for viable cells numbers. In the mineral water bottle systems studied by Jones et al. [26], surface roughness appeared to be most significant in determining adhesion, while surface hydrophobicity and electrostatic charge had no significant role.

4.2. Other factors

There has been some debate on higher bacterial counts that are generally found in PVC compared with those in glass bottles [5]. It was argued that the major cause of the lower colony counts of the same mineral water, bottled in mechanically cleaned glass than in plastic bottles, was due to the bacteriostatic effect of residual cleaning agents.

The storage temperature of the bottles has never been thoroughly studied. Usually the maximum bacterial density was observed in room temperature (about 20°C) stored samples. Nevertheless, storage at low temperatures, such as that of refrigeration (4–6°C), does not stop bacterial multiplication.

Photodegradation of dissolved organic matter is a common phenomenon. Thus the exposure time of recalcitrant organic substances in water samples to daylight, and moreover to sunlight, may again stimulate the growth of microorganisms since complexed substances, such as carbohydrates, fatty acids, and amino acids, may become bioavailable. Photochemical processes generate low molecular mass, readily biodegradable molecules from high molecular mass humic complexes [7].

The counts of cultivable bacteria that can be recovered from mineral waters also depend to a large degree on the culture methods used. Counting colonies of bacteria on rich nutrient media, as done in medical microbiology, has dominated past research for a long time. Fortunately from 1970 to the 1980s the concept of substrate shock (too much nutrients) has been successfully addressed [7]. Thus, when low-nutrient medium such as R2A is used for environmental samples, a significant increase in viable counts can be generally observed compared to the use of regular strength medium such as standard plate count agar [6]. R2A agar incubated at 20°C has proven to be especially suitable. There is dramatically less or even no bacterial growth at 37°C compared to 20°C [5]. It is possible that high plate counts at 37°C, such as staphylococci, coryneforms or Gram-positive bacilli, indicate allochthonous populations to the mineral water. These thermotrophic bacteria which can grow in mineral water at 42°C were below 10^3 l^{-1} [29]. The choice of incubation time is probably the most important factor for isolating bacteria from bottled mineral water because many of these organisms are slow growing. Thus, for species distribution studies it is important to incubate the cultures for longer periods (up to 14 days at 20°C has frequently been used) than for monitoring purposes (3 days).

4.3. Growth or resuscitation

It remains unclear whether the ultimate large population of culturable bacteria in mineral water is due to resuscitation of a large number of non-culturable dormant (VBNC) cells present in the water source or in the bottling system, or whether it is the result of cell division and growth of a few culturable cells initially present. Resuscitation is defined here as a reversal of the metabolic and physiological processes that result in non-culturability, i.e. the restoration of the ability of the cells to be culturable on media normally supporting growth of the organisms. Resuscitation would appear to be essential to the VBNC state if this is truly a survival strategy. Whereas the non-culturable state may in some way protect the cell against one or more environmental stresses, resuscitation of the cell would allow it to compete actively in the environment. However, according to Bogosian [30], recovery of culturable cells from a population of non-culturable cells, via a process of resuscitation, can be confounded by the presence of a low level of culturable cells, which can grow in response to the addition of nutrients and give the illusion of resuscitation. The data of Oger et al. [31] seem to demonstrate that the culturable population arising within 1 week of storage is derived from a large number of bacteria, in a 'minicell' state but stainable by AO ($\sim 10^3$ cells ml^{-1}) and, therefore, from resuscitation processes. Ferreira et al. [32] assume that such large populations of culturable bacteria are the result of growth from a very low number of organisms enumerated with ethidium bromide, initially present at the source and (or) the bottling system.

Compared to cultivation-based methods, nucleic acid probes currently allow the taxonomically most precise and quantitative description of microbial community structures. Over the last decade, rRNA-targeted probes have become a handy tool for microbial ecologists [33]. The fluorescence in situ hybridization (FISH) with rRNA-targeted probes leads to detect and identify bacteria even at a single cell level without prior cultivation and purification.

This method had been optimized [34,35] and applied to mineral water by combining it with membrane filtration (Fig. 1). Probes were used that were specific for the Bac-

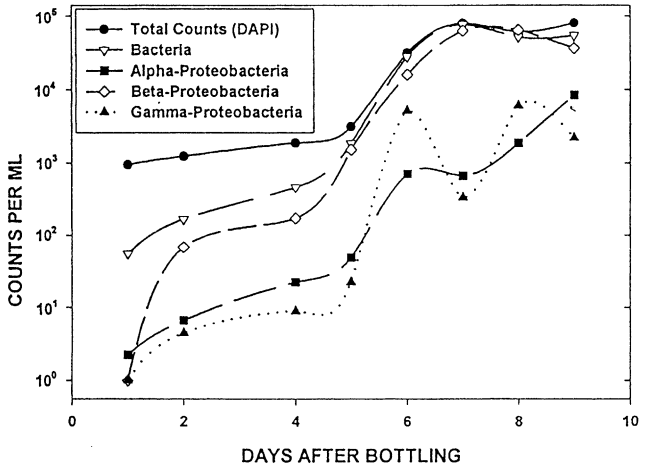

Fig. 1. Total counts and counts for active bacteria and bacterial subgroups in French uncarbonated NMW during 9 days after bottling (PET bottles). Total counts (number of bacteria stained by DAPI). Active bacteria, active α-, β-, γ-Proteobacteria (number of bacteria detected by respective Cy3-labelled rRNA-targeted probes) (from W. Beisker, personal communication).

teria, the α-subclass, β-subclass and the γ-subclass of Proteobacteria. The applied fluorescent probes were targeted to rRNAs and the number of cells detected with fluorescently labelled rRNA-targeted probes was called 'probe active count'. The development of the bacterial community in a PET bottled uncarbonated water sample was monitored during 9 days after bottling, using the FISH method and DNA staining with DAPI (W. Beisker, personal communication). (i) As measured by AODC, the number of bacterial cells increased from 1000 ml^{-1} to 8×10^4 within 7 days after PET-bottling (Fig. 1) which is similar to the others studies [22,31,32,36,37]. (ii) As only 5% of total counts (DAPI) were detected the first day by the Bacteria-specific probe, the number of physiologically active bacteria (viable and culturable) can be assumed to be significant while the plate count of still mineral waters is generally in the order of a few CFU per ml on the R2A medium (about 1–5 CFU ml^{-1}). This portion increases slowly up to day 5, then rapidly between days 5 and 7. It appears that the increase of total count might essentially be due to growing physiological by active bacteria that have been detected by the eubacterial probe. These results suggest that the apparent resuscitation was merely due to the growth of the culturable cells from day 1. (iii) The appearance of biphasic growth or a double growth cycle (diauxie) is typical of media that contain mixtures of substances. The first substrate will induce the synthesis of those enzymes required for its utilization and at the same time will repress the synthesis of enzymes required for second substrate. The latter enzymes are only produced when all of the first substrate has been metabolized. However, the recent results obtained for the kinetics of growth during 'mixed substrate growth' [38] suggest that simultaneous utilization of carbon sources will result at the low substrate concentrations present in these systems and that allows growth at very low concentrations of individual carbon substrates. (iv) The bacterial population in bottled mineral water is dominated by Proteobacteria and β-Proteobacteria were found to be the most abundant group of detected bacteria (see Section 5).

5. Microbial community

Community structure is generally considered to be related to the types of organisms present in an environment and to their relative proportions. For natural mineral waters all the data have been obtained, thus far, by culture dependent methods. The organisms most widely isolated from mineral water and representing major groups are shown in Table 1. These results were obtained in extensive studies by Schwaller and Schmidt-Lorenz [41], Bischofberger et al. [22], Manaia et al. [42], Guillot and Leclerc [43] and Vachée et al. [37]. The vast majority of the het-

Table 1
Major groups of bacteria isolated from natural mineral waters. +, less than 10% of isolates; ++, between 10% and 50%

Classification	Schwaller and Schmidt-Lorenz [40,41]	Bischofberger et al. [22]	Manaia et al. [42]	Leclerc et al. [37,43]
Proteobacteria γ-subclass				
Pseudomonas fluorescent spp.	++	++	++	++
Pseudomonas non-fluorescent spp.	++	+	++	+
Acinetobacter	++	+	+	+
Stenotrophomonas maltophilia	–	+	+	+
Proteobacteria β-subclass				
Alcaligenes	+	+	++	+
Comamonas acidovorans	+	–	++	+
Comamonas testosteroni	+	+	–	+
Acidovorax delafieldii	+	+	–	–
Paucimonas lemoignii	–	++	–	–
Proteobacteria α-subclass				
Brevundimonas diminuta	–	–	–	+
Brevundimonas vesicularis	–	–	–	+
Cytophaga–Flavobacterium	++	++	++	+
Arthrobacter, *Corynebacterium*	+	++	–	+

erotrophic bacteria of natural mineral waters can be classified in a restricted number of phylogenetic divisions. Bacteria belonging to the α-, β- and γ-subclasses of the Proteobacteria, and members of the *Cytophaga–Flexibacter–Bacteroides* phylum, are the most common bacteria isolated from bottled mineral water (Table 1). Many local and intermediate flow systems are well oxygenated and, thus, the predominant types of bacteria are aerobic.

5.1. Prosthecate bacteria

Prosthecate bacteria members of the Proteobacteria such as *Caulobacter* are characterized by an elongated, cylindrical appendage, designated a prosthecate. It is experimentally clear that these organisms are highly successful scavengers of very low concentrations of nutrients, and it has been suggested that competitive advantages for an oligotrophic mode of existence are due to efficient uptake of nutrients and the possession of a high surface area-to-volume ratio [39]. Given the oligotrophic conditions of most aquifer systems, *Caulobacter* should be widely distributed in groundwater samples. In fact the occurrence of prosthecate bacteria has rarely been reported in natural mineral waters, but these bacteria have not usually been sought because of their special medium requirements. It is, nevertheless, interesting to note the predominance of appendaged and (or) budding bacteria in all the springs examined in the survey of Gonzalez et al. [40]. *Caulobacter* was the most frequently isolated organism in both the bottled water and the water collected at the source. A large appendaged and budding bacterium similar to *Hyphomicrobium* or *Hyphomonas* was also recovered from some samples. However, it may be that a low tolerance to decreased oxygen concentration could limit the occurrence of prosthecate bacteria in groundwater habitats.

5.2. Pseudomonas, Acinetobacter, Alcaligenes

By far the most important members of the mineral water flora are fluorescent and non-fluorescent pseudomonad species (Table 1). The genus *Pseudomonas*, now restricted to rRNA group I according to Palleroni et al. [44], encompasses some genuine *Pseudomonas* species that display a genome and phenotypic relationship to the type species *P. aeruginosa*. However, it is important to note that the 'clinically relevant' *P. aeruginosa* (producing both pyocyanin and fluorescent pigment) is not a normal component of the microbial flora of natural mineral waters whereas fluorescent pseudomonads (producing only fluorescent pigment) are typical soil and subsurface environments.

In the two studies of Guillot and Leclerc [43] and Vachée et Leclerc [37] including 1350 strains of representative bacteria from mineral waters, the unidentified isolates reached about 80%. Many unclassified genomic groups were found to represent the following new species of the genus *Pseudomonas*: *P. veronii* [45], *P. rhodesiae* [46], *P. jensenii* [47], *P. mandelii* [47], *P. gessardii* [48], *P. migulae* [48], *P. brenneri* [49], *P. grimontii* [50]. Three new species, *P. libanensis* [51], *P. cedrella* [52] and *P. orientalis* [52], were also isolated from Lebanese springs. Thus, microflora of mineral waters should have a high fluorescent pseudomonads content. One reason pseudomonads are common in groundwaters is that they are extraordinarily versatile in the kinds of organic substrates on which they can grow. In addition, they do not require specific vitamins or amino acids and readily live on a number of different carbon sources. The adaptability of *Pseudomonas* and related bacteria makes them ideal candidates for colonizing groundwater systems where organic carbon compounds are largely limited to DOC leaching out of the soil zone above (local flow system) or present in the sediments as a primary carbon source (intermediate flow system).

The strains of the genera *Acinetobacter* and *Alcaligenes* were isolated in all studies in numbers that sometimes rivalled those of the genus *Pseudomonas* (Table 1). In decreasing order of importance, species of *Comamonas*, *Burkholderia*, *Ralstonia* and *Stenotrophomonas* were also isolated, followed by species of *Sphingomonas*, *Acidovorax*, *Brevundimonas* and *Paucimonas*.

5.3. Cytophaga, Flavobacterium, Flexibacter

It is not uncommon to observe yellow, orange, or brick-red colored colonies on agar plated with mineral water samples. Sometimes these form films that may cover the whole plate within a few days. In other cases, the colonies expand slowly or remain more or less compact. In few instances, rhizoid growth is also observed. Many of the strains produce flexirubin-type pigments in addition to carotenoids. These bacteria generally belong to the genera *Cytophaga*, *Flavobacterium* and *Flexibacter*, which are regularly isolated from most natural mineral waters, sometimes even as dominant populations [5]. These bacteria usually found in habitats rich in organic material can however adapt to the groundwater environment, but perhaps more readily to shallow aquifers (local flow systems) where dissolved oxygen concentrations are relatively high and relatively open to sources of nutrients from the surface or from the unsaturated zone.

5.4. Gram-positive bacteria

Gram-positive bacteria occurring in natural mineral waters have sometimes been reported to belong to 'arthrobacter-like' or 'coryneform-like' bacteria and more rarely to *Bacillus*, *Staphylococcus* and *Micrococcus* [5]. However, there is convincing evidence that they are derived from the bottling plant, since Gram-positive bacteria such as *Micrococcus* and *Staphylococcus* are common inhabitants on the skin and mucous membranes of humans [29]. All these bacteria may be part of the ambient microflora.

The distribution of Gram-positive bacteria is a critical issue in groundwater systems. Transmission electron microscopy showed, in fact, that about two-thirds of the bacterial cells from subsurface environments had Gram-positive cell walls, whereas isolation of microorganisms on culture medium revealed a preponderance of Gram-negative cells [2]. In addition to direct microscopic observation, biochemical techniques can also give an indication of the relative abundance of Gram-positive and Gram-negative microorganisms in samples. For example, the amount of ribitol, which is a part of teichoic acids of Gram-positive bacteria, is a rough measure of their relative abundance. Likewise, the abundance of Gram-negative bacteria can be estimated by the level of hydroxy fatty acids in the lipopolysaccharides.

The ability to form endospores when growing cells are subjected to nutritional deficiency or excessive heat or dryness is characteristic of some Gram-positive bacteria such as *Bacillus* and *Clostridium*. Endospores could be particularly well adapted to environments subjected to wide variations in water and low-nutrient conditions such as subsurface environments but, with some exceptions, species of *Bacillus* or *Clostridium* have not been reported widely from aquifer systems [2]. These observations indicate that spore formation per se might not be a major feature for bacteria inhabiting groundwater habitats.

5.5. Microbial diversity and specificity

Mineral water ecosystems, including those in aquifers, exhibit a high degree of phenotypic and genetic microbial diversity that cannot always be supported by species identification [2]. Phenotypic characteristics that rely on physiological activities have been shown to be less important for estimating bacterial diversity than genetic characteristics, because many metabolic traits may be induced or repressed by different environmental conditions. Restriction fragment length polymorphism (RFLP) patterns of rDNA regions (ribotyping), therefore, constitute a more reliable method for assessing genetic diversity within autochthonous bacterial associations of mineral water.

In our laboratory experiment on the fate of the bacterial flora at source, before and after bottling [37], we have isolated 890 strains from five springs (A–E) and observed 378 distinct ribotypes. A marked degree of heterogeneity was observed amongst ribopattern profiles, accounted for in each spring by the Simpson index of diversity [2]. This index ranged from 97.8% (spring A) to 99.4% (spring E). RFLP analysis detected a large number of polymorphisms combined with unequivocal band resolution in all groups, but particularly high in a set of isolates producing a fluorescent pigment (72 patterns for 174 strains). So, each spring should be characterized by a wide diversity of ribopattern profiles but most of these ribotypes reveal their own 'personal' property. Indeed amongst the observed patterns, only a few were common to one or several mineral waters. Spring A contains two isolates in common with spring B, three with spring C, five with spring D and one with spring E; spring B has two isolates in common with spring A, three with spring C, two with spring D, three with spring E, etc... Furthermore, the incidence of repeatedly recovered isolates before and after bottling varies from 52 to 90%, with an average of 70%. These data suggest that, within the ground-mineral water bacterial community, a high percentage of indistinguishable or closely related isolates (identical ribopatterns) of non-fermentative Gram-negative rod-shaped bacteria occurring in pristine mineral waters is retained during the bottling procedure and storage.

6. Assessing health risks from autochthonous bacteria

There are several approaches to detecting bacterial populations such as those autochthonous to mineral waters that could have significance in terms of public health but that are not known to be pathogenic. The methods available include animal model systems, epidemiological studies and search for virulence factors from bacterial isolates.

6.1. Animal model system

Axenic animals constitute a preferred choice in determining whether the autochthonous bacteria occurring in mineral water are able to adhere, penetrate and multiply in epithelial cells, or produce toxins or irritating substances causing tissue damage. A very stringent experiment was devised to compare the transit of an inoculum of several autochthonous strains in pure cultures and that of spores used as markers [53]. In spite of the presence of an equivalent number of *Pseudomonas* (strain P1) cells and of the inert marker in the inoculum, the maximum number of *Pseudomonas* in the feces was lower than that of the spores, and the former disappeared from the feces more rapidly than the latter. Thus, a partial destruction of *Pseudomonas* P1 was shown during its transit through the digestive tract. Other strains that are predominant in water, i.e. *Pseudomonas* spp. or *Acinetobacter* spp., provided similar results.

6.2. Randomized trial in infants

The safety of water used for the preparation of baby feeding bottles is universally recognized as essential. In the past, mineral water bottled in glass was used. Since 1970, PVC has been used for bottling and some people have wondered about the modifications in the microbial populations that may have resulted from using water bottled in PVC, as well as effects on the health of babies. To answer this question, a study [54] was carried out, including 30 babies fed with milk reconstituted from powder with nat-

ural mineral water in PVC bottles, another 30 receiving milk made with the same mineral water which had previously been pasteurized in glass bottles. The test was double-blind. All babies were carefully selected. In no case was it possible to isolate mineral water-derived bacteria from nasopharyngeal swab samples, 1 or 2 h after drinking the milk. Nor was there evidence of digestive tract colonization when examining stool samples. From a clinical point of view, no differences could be found between the two groups. In no case was evidence obtained justifying the suspension of milk feeding.

6.3. Virulence characteristics of bacteria

Several studies have been carried out to test the invasive or cytotoxic activity of the bacterial flora in drinking water on cultured cell lines [55–57]. In all cases a small percentage (1–2%) of bacteria examined were cytotoxic. In the study of Payment et al. [56] a high percentage of the cytotoxic bacteria isolates belonged to the genus *Bacillus*.

A study was conducted in our laboratory to determine the virulence characteristics of natural mineral water bacteria. The tests selected determine the ability of bacteria to attach, invade and injure Hep-2 cells. The method used was the one described by Edberg et al. [57]. A total of 240 representative strains isolated from five French springs were selected, including *P. fluorescens* and several new species, such a *P. rhodesiae* [46], *P. veronii* [45], *P. gessardii* and *P. migulae* [48], *P. jessenii*, *P. mandelii* [47], *P. libaniensis*[51], *P. cedrella* and *P. orientalis* [52]. Results showed that none of the bacteria studied is capable of growing and attaching on Hep-2 cells or producing cytotoxin at a temperature of 37°C. The detection of bacterial activity in one or several of the tests for putative virulence factors may be useful in showing potential health hazards posed by bacteria isolated from potable water. Nevertheless, the exact relationship between putative virulence factors and their potential health effects remains to be investigated.

Overall experimental and epidemiological data show that autochthonous bacteria in natural mineral waters have never brought about detectable pathological disorders in human or animals and, in vitro, are incapable of directly damaging human cells in tissue culture. Since the existence of European regulations dating from 1980 [58], no outbreak or single case of disease due to the consumption of natural mineral water has been recorded in the literature, or by the health authorities of the countries within the European Community.

7. Assessment and management of microbial health risks

7.1. Identifying microbial hazards in drinking water

A large variety of bacterial, viral, and protozoan pathogens are capable of initiating waterborne infections. (i) There are primarily the enteric bacterial pathogens including classic agents such as *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp. and newly recognized pathogens from fecal sources like *Campylobacter jejuni* and enterohemorrhagic *E. coli*. The survival potential of these bacteria increases in biofilms and due to their stages as VBNC cells. (ii) Several new bacterial pathogens such as *Legionella* spp., *Aeromonas* spp., *P. aeruginosa* and *Mycobacterium avium* have a natural reservoir in the aquatic environment and soil. These organisms are introduced from the surface water into the drinking water system usually in low numbers. They may survive and grow within the distribution system biofilm. (iii) More than 15 different groups of viruses, encompassing more than 140 distinct types, can be found in the human gut. These enteric viruses are excreted by patients and find their way into sewage. Hepatitis A and E viruses cause illness (hepatitis) unrelated with gut epithelium. Another specific group of viruses has been incriminated as a cause of acute gastroenteritis in humans; it includes rotavirus, calicivirus, the most notorious being Norwalk virus, astrovirus and some enteric adenovirus. These viruses cannot grow in the receiving water and may only remain static in number or die-off. (iv) The most prevalent enteric protozoa, associated with waterborne disease, include *Giardia lamblia* and *Cryptosporidium parvum*. In addition, protozoa like *Cyclospora*, *Isospora* and many microsporidian species are emerging as opportunist pathogens and may have waterborne routes of transmission. Like viruses, protozoa cannot multiply in the receiving waters. With the exception of *Salmonella*, *Shigella* and hepatitis A virus, all the other organisms can be so-called 'new or emerging pathogens'.

There are a number of reasons for the emergence of these new pathogens, analyzed in every detail by Szewzyck et al. [59], including high resistance of viruses and protozoan cysts, a lack of identification methods for viruses, change in habit of water use (*Legionella*), subpopulations at risk. Another striking epidemiological feature is the low number of bacteria that may trigger disease. The infectious dose of *Salmonella* is in the range of 10^7–10^8 cells while only around 100 cells are required to cause clinical illness with *E. coli* O157:H7 and *Campylobacter* [59]. The infective dose of enteric viruses is low, typically in the range of 1–10 infectious units; it is about 10–100 oocysts for *Cryptosporidium* [60].

7.2. Concerning certain characteristics of natural mineral water

All microbial hazards occurring in drinking water from distribution systems must be taken into account in the case of natural mineral water. However, it is appropriate to consider some specific characteristics of mineral waters, related to microbial risk assessment. (i) In terms of public health, no outbreak or single case of disease due to the

consumption of natural mineral water, in line with European microbiological standards, has been recorded. Other epidemiological data including cohort study in infants, animal tests and cell tests have never showed adverse effects (see Section 6). (ii) In the past decade, many outbreaks attributed to protozoan or viral agents have been reported in conventionally treated water supplies, all of which met coliform standards. Viruses have been shown to persist longer in these waters than fecal coliforms and many are more resistant to water and wastewater treatment processes. A similar situation exists for protozoan cysts. These findings repeatedly suggest the inadequacy of treatment processes for safe water and the inadequacy of coliforms as indicators. Microbiologically, groundwaters tend to be better quality than surface waters and, consequently, require no treatment or less intensive treatment. However, many groundwater systems are under the direct influence of surface water, and thus vulnerable to fecal contamination, while natural mineral waters are recognized as not being vulnerable following a strict recognition procedure that requires a few years evidence of stability in physical and chemical characteristics, and microbiologically wholesomeness. (iii) A factor that might promote the growth of bacteria in water distribution or mineral water after bottling is the availability of organic carbon (DOC) or other limiting compounds. The most distinctive factor of mineral waters might be the very low amount of DOC with an available fraction and its identifiable compounds such as labile amino acids, carbohydrates and carboxylic acids [61]. Organic substances in the distribution network water originate from the raw water (generally surface water) used for its production and from materials (pipematerials, lubricants, joints, sealants, etc...) that may release biodegradable compounds [62]. These nutrients are a major factor for pipe colonizing heterotrophic bacteria. The so-formed biofilms are capable to retain pathogens including environmental pathogens (*Legionella*, *M. avium*), viruses and protozoa entering a distribution network. (iv) Mineral water bacterial communities, identified by culture or with specific probes, are primarily aerobic Gram-negative rods. These bacteria belong to three α-, β- and γ-proteobacterial groups as well as to the phylum of *Flavobacterium–Cytophaga*. In contrast, the general population in water supplies includes many Gram-negative and Gram-positive bacteria, sporeformers, acid-fast bacilli, free-living amoebae and nematods, opportunistic fungi and yeasts [62]. Many authors have observed antibacterial activity by autochthonous flora of mineral waters; however the issue is widely debated [5]. (v) For mineral water sources claiming to be protected, an inherent feature is that the physical and chemical nature of water is constant over time. Therefore, simple measurements such as temperature, ionic strength, anions, cations and trace elements have great meaning in sampling source water, whereas they would have little meaning when sampling tap water.

7.3. Assessment of microbial risks

The view on the microbiological safety of drinking water is changing. The demand for the total absence of any pathogenic organism is no longer significant in light of the new pathogens, some of which are capable of growing in drinking water systems. According to the new European Union Council directive 98/83/EC [63], water for human consumption must be free from any microorganisms and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health. To deal with this issue, the U.S. Environmental Protection Agency for the first time used a microbial risk assessment approach. It has been defined that an annual risk of 10^{-4} (one infection per 10 000 consumers $year^{-1}$) should be acceptable for diseases acquired through potable water, this value being close to the annual risk of infection from waterborne disease outbreaks in the United States (4×10^{-3}) [64].

Microbiological risk assessment is a major tool for decision making in the regulatory area. The problem is, however, that the key data to perform this assessment are mostly missing. Few epidemiological studies associating the incidence of disease to the pathogen densities have been reported. Several outcomes, from asymptomatic infection to death, are possible through exposure to microbes. The issue of dose–response relationships is particularly striking [59]: these relationships are only available for a few pathogens; when infectious doses are low as is the case for some viruses and protozoan cysts, the calculated tolerable concentrations are also low and monitoring of these pathogens in drinking water becomes impracticable.

Natural mineral waters are subject to the general rules laid down by council directive 80/777/EEC [58]. At source and when sold, a natural mineral water must be free from parasites and pathogenic microorganisms. These requirements are therefore distinct from tap water.

7.4. Management of microbial risks

7.4.1. Heterotrophic plate counts (HPC)

HPC measurements have been used to gain better information on the effects of water treatment processes and distribution on the bacteriological quality of drinking water. Various methods and the application of HPC monitoring have been analyzed in considerable depth by Reasoner [65]. In certain epidemiological studies reported by Payment [66], there is a debate on the potential negative impact to human health from the consumption of treated water containing high HPC levels of bacteria.

Natural mineral water cannot be subjected to any type of disinfection that modifies or eliminates their biological components; therefore, they always contain the bacteria that are primarily a natural component of these waters [5]. It is also clearly stated that, after bottling, the recov-

erable bacterial counts should only result from the normal increase of bacteria present in the source. The above studies (see Section 6) have not been capable of identifying any microbiological risk from examined bottled mineral waters. To date, there has been no association between human disease and the natural bacteria found in natural mineral water.

Measurement of HPC in bottled mineral water is useful for several reasons [67]. Firstly it proves that no disinfection has occurred. Secondly, it helps to ensure that, from the spring to the finished product, no major quantitative changes have occurred in the microbial status of the water. Indeed modification from counts normally found at a particular location may give an early warning of significant microbial alteration.

The bacterial species that make up HPC in natural mineral water are psychrotrophic. In the study by Reasoner and Geldreich [68] concerning treated distribution water, incubation at 20°C yielded the highest counts on all media when incubation was extended to 12–14 days, whereas 28°C appeared to be the best temperature from day 2 through day 6 of incubation. The 37°C plate count was believed to give an indication of the presence of rapid growing bacteria more likely to be related to pathogenic or fecal types that might be present from sewage pollution. It can be stated that the measurement at 37°C is unsuitable and unnecessary to determine inadequate processing for safety because there are many appropriate indicators capable of doing this, including the indicators of fecal contamination.

7.4.2. Marker organisms and enteric pathogens

In the microbiological monitoring of water and foods, Ingram [69] introduced the distinction between 'index organisms' for markers whose presence indicates the possible occurrence of ecologically similar pathogens, and 'indicator organisms' for those whose presence points to inadequate processing for safety. In short, index markers indicate a potential health risk, whereas indicators reveal process failure. The terms 'indicator of fecal contamination' (index) and 'indicator of quality' (indicator) are also commonly used. *E. coli* is now the sole recognized indicator of fecal contamination, being a direct public health threat [70]. The other indicators termed 'of quality' include coliforms other than *E. coli*, commonly fecal streptococci, *P. aeruginosa* and sulfite-reducing anaerobes: in the case of treated drinking water they demonstrate treatment effectiveness and water quality characterization in the distribution system (biofilm development); in the case of untreated groundwater (mineral water) they indicate possible deficiency in natural hydrogeological protection mechanisms (indicator of vulnerability). No indicators can indicate the occurrence of environmental pathogens such as *Legionella* or *P. aeruginosa*.

Innovating taxonomic approaches in the bacteriology of the coliform group and comprehensive studies of their habitats have allowed the ecological positioning of coliforms in one of the three following groups [71]: (i) the thermotrophic and true fecal *E. coli*; (ii) the thermotrophic and ubiquitous coliforms (e.g. *Klebsiella pneumoniae*, *Enterobacter cloacae*), which may form part of the intestinal flora of man and warm-blooded animals, but also occur in the natural environment; and (iii) psychrotrophic, purely environmental coliforms (e.g. *Serratia fonticola*, *Rahnella aquatilis*), which proliferate in polluted or pristine waters and mostly originate from vegetable or small animal sources. From a public health point of view both the ubiquitous and environmental groups are grouped with quality indicators. The controversy over the value of 'fecal coliform' or 'thermotolerant coliform' as fecal indicators, associated with the heterogeneity of the group, has led to the suggestion that the term 'fecal coliform' or 'thermotolerant coliform' should be redefined to be synonymous with *E. coli* [71].

Basically, an acceptable indicator of pathogens, such as *E. coli*, must only have two attributes. An acceptable indicator must be present when the pathogens are present, and it must be easy to detect and to quantify. It is sometimes important, but not imperative for the protection of the public health, that the indicator is absent when the pathogen is absent. The most significant change over the last two decades is the general recognition that the coliform test including *E. coli* in treated water supply is not so much a measure of sanitary significance but more an indication of treatment effectiveness. Coliform bacteria as well as other bacterial indicators are easily captured then inactivated in conventional treatment processes but the more resistant enteric viruses and protozoan pathogens are not. It is now considered that the use of *E. coli* may be the most appropriate to indicate the presence of enteric bacterial pathogens and that viruses and protozoan pathogens must be analyzed separately [72].

With natural mineral waters that are untreated, the problem of a relationship existing between marker organisms and pathogens must be discussed specifically, as this relationship is governed by key factors and processes that control the mobility and fate of suspended microbes in soil and groundwater environments. The first category of factors focuses primarily on characteristics of the microbes such as size, adhesion and inactivation or die-off rate. The second category pertains to abiotic factors such as porous medium characteristics, filtration effects and water flow. The implication of microbial transport relative to the safety of groundwater has been closely analyzed by Robertson et al. [73] and Newby et al. [74]. In general, the larger the suspended microorganism, the more readily it will be physically filtered by the subsurface material. Thus, parasite cysts or oocysts, such as those of *Giardia* and *Cryptosporidium*, are relatively large and thus much more readily filtered than viruses and bacteria. However, the die-off rate of *E. coli* through transport in subsurface environments is certainly higher than that of *Giardia* or

Cryptosporidium cysts. Taking into account the half-life of *E. coli*, conservatively estimated to be at least 8 days under groundwater conditions, Edberg et al. [75] recommended the use of *E. coli* as indicator of fecal protozoan.

The relatively high mobility of viruses in subsurface material is primarily due to their smaller size, lower inactivation or die-off rates, and physical properties compared with bacteria. Enteric viruses have been detected in many groundwater supplies, usually those in close proximity to surface water or septic tanks (vulnerable groundwater). Thus, viral contamination of groundwater is of special concern. There is no absolute correlation between bacterial indicators and enteric viruses due to the essentially unpredictable behavior of viruses. Coliphages do not yet fulfil enough of the criteria to be reliably employed [76]. Viral pathogens including hepatitis virus A, enterovirus and calicivirus should be detectable by a combination of cell culture and molecular methods (awaiting validation by international groups of experts). Actually there is a large discrepancy in the results of studies in comparing infectivity, molecular, and combined methods [77,78]. The report on Norwalk-like virus sequences detected in bottled mineral waters shows the difficulties in choosing appropriate methods and the very high risk of contamination involved [79].

The indicators of quality for natural mineral waters are ubiquitous bacteria, most often being common to soil and vegetation and unrelated to plain fecal contamination. In the absence of *E. coli*, their presence in a water sample does not indicate an imminent health threat. However, they are very sensitive indicators of surface contamination and can appear as the first agents of water quality change. Their occurrence in mineral water at source and after bottling should be limited to a low number of events and a study to find the origin has to be conducted. A single-sampling procedure allows no flexibility in the interpretation of positive findings. There are objections against such a procedure in the sense that it may become technologically impracticable. A three class-sampling plan that incorporates so-called tolerances as used for microbiological safety requirements is much more rational [80]. The recognition process for a new source of natural mineral water requires a few years evidence of stability that must be demonstrated by continuous monitoring at the source of physico-chemical and microbiological parameters listed in European directives [58]. Monitoring should include periodic sampling of water (at least four times a year) at the collection point, with analysis for new pathogens such as *Cryptosporidium* and enteric viruses.

In the light of epidemiological and ecological data it appears that the combination of two categories of markers, i.e. indicators of fecal contamination and indicators of vulnerability, may be the most appropriate for characterization of a microbiological safe natural mineral water. However, taking into account the numerous outbreaks occurring in the world, especially in the U.S. it is recommended to intensify microbiological monitoring in order to detect regularly (for instance, once a year), but not routinely, viral and protozoan pathogens.

7.4.3. Pathogens growing in water

There is a variety of environmental opportunistic human pathogens that can pass through water treatment barriers at very low densities and take advantage of selected sites in the water supply systems to colonize. They are typical biofilm organisms that grow at the periphery of distribution systems (long pipes leading to dead ends) and throughout the pipe network where the water can be stagnant. The most important organisms to consider are *P. aeruginosa*, *Aeromonas*, *Legionella* and *Mycobacterium* complex. Their significance in treated drinking water has been discussed in detail by Szewzyck et al. [59].

It has been shown recently [81] that members of the genus *Mycobacterium* are present in drinking waters; however, the numbers and frequencies of recovery of *M. avium* and *M. intracellulare* are usually low. The occurrence of non-tuberculous mycobacteria such as *M. avium*, *M. kansasii* or *M. intracellulare* has never been reported for mineral water samples [82]. *Aeromonas* are widespread in surface waters. Their presence in sediment accumulated in pipelines in the water supply is an indication of biofilm development. Their significance in drinking water relative to the occurrence of gastrointestinal infections is a much debated question. *Aeromonas* spp. are sometimes able to contaminate mineral water in low numbers for the same reason as coliforms or *P. aeruginosa*. Their significance is the same as that of quality indicators.

It is now well established that legionellae are ubiquitous in engineered water supplies, plumbing systems of hospitals and other large buildings, being an important cause (5–15%) of community-acquired and of hospital-acquired pneumonia [83]. Epidemiological and genetic studies demonstrated that environmental amoebae have acted as an evolutionary incubator for the emergence of *Legionella pneumophila* as an opportunistic pathogen for human [84]. The occurrence of *Legionella* within biofilms and its ability to enter a VBNC state contribute to its survival [83]. *Legionella* spp. are being looked at as bacteria able to contaminate mineral water. However, the occurrence of *Legionella* has never been reported from mineral water either at source or in bottles. But the problem is highly relevant for the use of mineral water in hydrothermal areas where warm spa water can promote the growth of legionellae [85]. Here various care categories for patients, including nebulizers, hot whirlpool spa, baths or other aerosols generating mechanical devices, can increase the risk of acquiring Legionnaires' disease. Risk assessment has important implications for the maintenance of adequate standards of hygiene, bacteriological monitoring and clinical surveillance in these establishments.

P. aeruginosa is an ubiquitous environmental bacterium. It can be isolated, often in high numbers, in common

food, especially vegetables. Its presence is constant in surface waters and sometimes at low levels in drinking water. Other than certain specific hosts at risk, the general population is resistant to infection with *P. aeruginosa* [86]. Since *P. aeruginosa* is capable of growing abundantly in the purest of fresh waters and since it has major opportunistic pathogen capability, its occurrence in natural mineral water should be limited as far as possible. So there are two reasons to monitor *P. aeruginosa* in mineral water: on one hand as an indicator of vulnerability and/or poor control of the bottling environment, on the other hand as an opportunistic pathogen.

8. Conclusion

Natural mineral water can be clearly distinguished from drinking water in distribution networks by physical, chemical and microbiological characteristics. From its origin, natural mineral water naturally contains bacterial populations in starvation–survival state, mainly entering a VBNC state with only few parts being culturable. After bottling, in the absence of any disinfection treatment, the revivable total colony count may only be that resulting from the normal increase in the bacteria content which it had at source.

With natural mineral waters that are untreated, the problem of risk management must be discussed specifically. Measurement of HPC proves that no disinfection and no major quantitative changes have occurred. The detection of indicator of fecal contamination *E. coli* may be the most appropriate to reveal the presence of enteric bacterial pathogens and possibly fecal protozoan. The indicators of quality such as coliforms other than *E. coli*, *P. aeruginosa* are sensitive indicators of surface contamination. In the absence of consensual indicator for pathogenic virus and protozoa, it is recommended to search separately and regularly for viral and protozoan agents.

The significance of *Aeromonas* in drinking water is a much debated question. The occurrence of *M. avium* complex organisms has never been reported in mineral water samples. *P. aeruginosa* can be monitored on the one hand as an indicator of vulnerability and on the other hand as opportunistic pathogen, its quantity should be limited as far as possible. The occurrence of *Legionella* has never been reported in mineral water either at source or in a bottle. The problem is relevant for the use of mineral water in hydrothermal areas where warm spa water or aerosols generating devices can increase the risk of infecting *Legionella* spp.

References

[1] Madsen, E.L. and Ghiorse, W.C. (1993) Groundwater microbiology: subsurface ecosystem processes. In: Aquatic Microbiology: an Ecological Approach (Ford, T.E., Ed.), pp. 167–213. Blackwell Scientific Publications, Boston, MA.
[2] Chapelle, F.H. (1993) Ground-water Microbiology and Geochemistry, 424 pp. Wiley, New York.
[3] Maier, R.M. and Pepper, I.L. (2000) Terrestrial environments. In: Environmental Microbiology (Gerba, C.L., Maier, R.M. and Pepper, I.L., Eds.), pp. 61–89. Academic Press, London.
[4] Paul, J.H. (1993) The advances and limitations of methodology. In: Aquatic Microbiology: an Ecological Approach (Ford, T.E., Ed.), pp. 15–47. Blackwell Scientific Publications, London.
[5] Leclerc, H. and da Costa, M.S. (1998) The microbiology of natural mineral waters. In: Technology of Bottled Water (Senior, D.A.G. and Ashurst, P., Eds.), pp. 223–274. Sheffield Academic Press, Sheffield.
[6] Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7.
[7] Morita, R.Y. (1997) Bacteria in Oligotrophic Environments. Starvation–Survival Lifestyle, 529 pp. Chapman and Hall, New York.
[8] Morita, R.Y. (1982) Starvation–survival of heterotrophs in the marine environment. Adv. Microbiol. Ecol. 6, 117–198.
[9] Matin, A. (1992) Physiology, molecular biology and applications of the bacterial starvation response. J. Appl. Bacteriol. Symp. Suppl. 73, 49S–57S.
[10] McCann, M.P., Kidwell, J. and Matin, A. (1992) Microbial starvation survival, genetics. In: Encyclopedia of Microbiology, Vol. 3, pp. 159–171. Academic Press, London.
[11] Mason, C.A. and Egli, T. (1993) Dynamics of microbial growth in the decelerating and stationary phase of batch culture. In: Starvation in Bacteria (Kjelleberg, S., Ed.), pp. 81–103. Plenum Press, New York.
[12] Nyström, T. (1993) Global systems approach to the physiology of the starved cell. In: Starvation in Bacteria (Kjelleberg, S., Ed.), pp. 129–151. Plenum Press, New York.
[13] Matin, A. (1991) The molecular basis of carbon starvation-induced general resistance in *E. coli*. Mol. Microbiol. 5, 3–11.
[14] Moyer, C.L. and Morita, R.Y. (1989) Effect of growth rate and starvation–survival on the viability and stability of a psychrophilic marine bacterium. Appl. Environ. Microbiol. 55, 1122–1127.
[15] Dow, C.S., Whittenbury, R. and Carr, N.G. (1983) The 'shut down' or 'growth precursor' cell–an adaptation for survival in a potentially hostile environment. Symp. Soc. Gen. Microbiol. 34, 187–247.
[16] Jones, C.R., Chamberlain, A.H.L. and Adams, M.R. (1999) An investigation of the presence of ultramicrocells in natural mineral water. Lett. Appl. Microbiol. 28, 275–279.
[17] Oliver, J.D. (1993) Formation of viable but nonculturable cells. In: Starvation in Bacteria (Kjelleberg, S., Ed.), pp. 239–272. Plenum Press, New York.
[18] Kurath, G. and Morita, R.Y. (1983) Starvation–survival physiological studies of a marine *Pseudomonas* sp.. Appl. Environ. Microbiol. 45, 1206–1211.
[19] Roszak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51, 365–379.
[20] Nystrom, T. (2001) Not quite dead enough: on bacterial life, culturability, senescence, and death. Arch. Microbiol. 176, 159–164.
[21] Buttiaux, R. and Boudier, A. (1960) Comportement des bactéries autotrophes dans les eaux minérales conservées en récipients hermétiquement clos. Ann. Inst. Pasteur Lille 11, 43–52.
[22] Bischofberger, T., Cha, S.K., Schmitt, R., König, B. and Schmidt-Lorenz, W. (1990) The bacterial flora of non-carbonated, natural mineral water from the springs to reservoir and glass and plastic bottles. Int. J. Food Microbiol. 11, 51–72.
[23] Fred, E.B., Wilson, F.C. and Davenport, A. (1924) The distribution and significance of bacteria in Lake Mendota. Ecology 5, 322–339.
[24] ZoBell, C.E. and Anderson, D.Q. (1936) Observations on the multiplication of bacteria in different volumes of stored sea water and the

influence of oxygen tension and solid surfaces. Biol. Bull. 71, 324–342.
[25] Heukelekian, H. and Heller, A. (1940) Relation between food concentration and surface for bacterial growth. J. Bacteriol. 40, 547–558.
[26] Jones, C.R., Adams, M.R., Zhdan, P.A. and Chamberlain, A.H.L. (1999) The role of surface physicochemical properties in determining the distribution of the autochthonous microflora in mineral water bottles. J. Appl. Microbiol. 86, 917–927.
[27] Jayasekara, N.Y., Heard, G.M., Cox, J.M. and Fleet, G.H. (1999) Association of micro-organisms with the inner surfaces of bottles of non-carbonated mineral waters. Food Microbiol. 16, 115–128.
[28] Davey, M.E. and O'Toole, G.A. (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64, 847–867.
[29] Leclerc, H., Mossel, D.A.A. and Savage, C. (1985) Monitoring non-carbonated (still) mineral waters for aerobic colonization. Int. J. Food Microbiol. 2, 341–347.
[30] Bogosian, G., Morris, P.J.L. and O'Neil, J. (1998) A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. Appl. Environ. Microbiol. 64, 1736–1742.
[31] Oger, C., Hernandez, J.F., Delattre, J.M., Delabroise, A.H. and Krupsky, S. (1987) Etude par épifluorescence de l'évolution de la microflore totale dans une eau minérale embouteillée. Water Res. 21, 469–474.
[32] Ferreira, A.C., Morais, P.V. and da Costa, M.S. (1993) Alterations in total bacteria, iodonitrophenyltetrazolium (INT)-positive bacteria, and heterotrophic plate counts of bottled mineral water. Can. J. Microbiol. 40, 72–77.
[33] Amann, R. and Ludwig, W. (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol. Rev. 24, 555–565.
[34] Wallner, G., Amann, R. and Beisker, W. (1993) Optimizing fluorescent in situ hybridization of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms. Cytometry 14, 136–143.
[35] Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.
[36] Morais, P.V. and da Costa, M.S. (1990) Alterations in the major heterotrophic bacterial populations isolated from a still bottled mineral water. J. Appl. Bacteriol. 69, 750–757.
[37] Vachée, A., Vincent, P., Struijk, C.B., Mossel, D.A.A. and Leclerc, H. (1997) A study of the fate of the autochthonous bacterial flora of still mineral waters by analysis of restriction fragment length polymorphism of genes coding for rRNA. Syst. Appl. Microbiol. 20, 492–503.
[38] Kovarova-Kovar, K. and Egli, T. (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol. Mol. Biol. Rev. 62, 646–667.
[39] Morgan, P. and Dow, C.S. (1985) Environmental control of cell-type expression in prosthecate bacteria. In: Bacteria in their Natural Environments (Fletcher, M. and Floddgate, G.D., Eds.), pp. 131–169. Academic Press, London.
[40] Gonzalez, C., Gutierrez, C. and Grande, T. (1987) Bacterial flora in bottled uncarbonated mineral drinking water. Can. J. Microbiol. 33, 1120–1125.
[41] Schwaller, P. and Schmidt-Lorenz, W. (1980) Flore microbienne de quatre eaux minérales non gazéifiées et mises en bouteilles. I. Dénombrement de colonies, composition grossière de la flore, et caractères du groupe des bactéries Gram négatif pigmentées en jaune. Zent.bl. Bakt. Hyg. I Abt. Orig. C1, 330–347.
[42] Manaia, C.M., Nunes, O.C., Morais, P.V. and da Costa, M.S. (1990) Heterotrophic plate counts and the isolation of bacteria from mineral waters on selective and enrichment media. J. Appl. Bacteriol. 69, 871–876.
[43] Guillot, E. and Leclerc, H. (1993) Bacterial flora in natural mineral waters: characterization by ribosomal ribonucleic acid gene restriction patterns. Syst. Appl. Microbiol. 16, 483–493.
[44] Palleroni, N.J. (1984) Genus, I. *Pseudomonas* Migula 1894. In: Bergey's Manual of Systematic Bacteriology, Vol. 1 (Krieg, N.R. and Holt, J.G., Eds.), pp. 141–199. Williams and Wilkins, Baltimore, MD.
[45] Elomari, M., Coroler, L., Hoste, B., Gillis, M., Izard, D. and Leclerc, H. (1996) DNA relatedness among *Pseudomonas* strains isolated from natural mineral waters and proposal of *Pseudomonas veronii* sp. nov.. Int. J. Syst. Bacteriol. 46, 1138–1144.
[46] Coroler, L., Elomari, M., Hoste, B., Gillis, M., Izard, D. and Leclerc, H. (1996) *Pseudomonas rhodesiae* sp. nov., a new species isolated from natural mineral waters. Syst. Appl. Microbiol. 19, 600–607.
[47] Verhille, S., Baida, N., Dabboussi, F., Izard, D. and Leclerc, H. (1999) Taxonomic study of bacteria isolated from natural mineral waters: proposal of *Pseudomonas jessenii* sp. nov. and *Pseudomonas mandelii* sp. nov.. Syst. Appl. Microbiol. 22, 45–58.
[48] Verhille, S., Baida, N., Dabboussi, F., Hamze, M., Izard, D. and Leclerc, H. (1999) *Pseudomonas gessardii* sp. nov. and *Pseudomonas migulae* sp. nov., two new species isolated from natural mineral waters. Int. J. Syst. Bacteriol. 49, 1559–1572.
[49] Baida, N., Yazourh, A., Singer, E. and Izard, D. (2000) *Pseudomonas brenneri* sp. nov., a new species isolated from natural mineral waters. Res. Microbiol. 152, 493–502.
[50] Baida, N., Yazourh, A., Singer, E. and Izard, D. (2000) *Pseudomonas grimontii* sp. nov., a new species isolated from natural mineral waters. Int. J. Syst. Bacteriol., in press.
[51] Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D. and Leclerc, H. (1999) *Pseudomonas libanensis* sp. nov., a new species isolated from Lebanese spring waters. Int. J. Syst. Bacteriol. 49, 1091–1101.
[52] Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D. and Leclerc, H. (1999) Taxonomic study of bacteria isolated from Lebanese spring waters: proposal for *Pseudomonas cedrella* sp. nov. and *P. orientalis* sp. nov.. Res. Microbiol. 150, 303–316.
[53] Ducluzeau, R., Dufresne, S. and Bochand, J.M. (1976) Inoculation of the digestive tract of axenic mice with the autochthonous bacteria of mineral water. Eur. J. Appl. Microbiol. 2, 127–134.
[54] Leclerc, H. (1990) Les qualités bactériologiques de l'eau minérale d'Evian. In: L'eau Minérale d'Evian, pp. 24–27. S.A. des Eaux Minérales d'Evian, Evian.
[55] Lye, D.J. and Dufour, A.P. (1991) A membrane filter procedure for assaying cytotoxic activity in heterotrophic bacteria isolated from drinking water. J. Appl. Bacteriol. 70, 89–94.
[56] Payment, P., Coffin, E. and Paquette, G. (1994) Blood agar to detect virulence factors in tap water heterotrophic bacteria. Appl. Environ. Microbiol. 60, 1179–1183.
[57] Edberg, S.C., Kops, S., Kontnick, C. and Escarzaga, M. (1997) Analysis of cytotoxicity and invasiveness of heterotrophic plate count bacteria (HPC) isolated from drinking water on blood media. J. Appl. Microbiol. 82, 455–461.
[58] EC (1980) Council Directive relating to the quality of water intended for human consumption (80/778/EEC). Off. J. Eur. Community L229, 11–29
[59] Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer, K.H. (2000) Microbiological safety of drinking water. Annu. Rev. Microbiol. 54, 81–127.
[60] Meinhardt, P.L., Casemore, D.P. and Miller, K.B. (1996) Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. Epidemiol. Rev. 18, 118.
[61] Lacoste, T. (1992) La matière organique des eaux thermales: caractérisation par des méthodes globales et spécifiques et évolution lors des traitements de désinfection. Thèse de Doctorat d'Université, Poitiers.
[62] Geldreich, E.E. (1996) Microbial quality of water supply in distribution systems. Lewis Publishers, CRC Press, Boca Raton, FL.
[63] EC (1998) Council Directive relating to the quality of water intended

for human consumption (98/83/EEC). Off. J. Eur. Community. L330, 32–54.
[64] Gerba, C.P. (2000) Risk assessment. In: Environmental Microbiology (Gerba, C.P., Maier, R.M. and Pepper, I.L., Eds.), pp. 557–571. Academic Press, London.
[65] Reasoner, D.J. (1990) Monitoring heterotrophic bacteria in potable water. In: Drinking Water Microbiology (McFeters, G.A., Ed.), pp. 452–477. Springer Verlag, New York.
[66] Payment, P., Richardson, L., Siemiatycki, J., Dewar, R., Edwardes, M. and Franco, E. (1991) A randomized trial to evaluate the risk of gastrointestinal disease due to consumption of drinking water meeting current microbiological standards. Am. J. Public Health 81, 703.
[67] Moreau, A. (2001) Surveillance microbiologique des eaux minérales naturelles. Bull. Soc. Fr. Microbiol. 16, 13–19.
[68] Reasoner, D.J. and Geldreich, E.E. (1979) A new medium for the enumeration and subculture of bacteria from potable water. In: Abstract of the Annual Meeting of the American Society for Microbiology, p. 180. American Society for Microbiology, Washington, DC.
[69] Ingram, M. (1977) The significance of index and indicator organisms in foods. Presented at Int. Symp. IUMS Comm. Food Microbiol. Hygiene, 10th, Szczecin, Poland. Lancet 2, 1425.
[70] Edberg, S.C., Rice, E.W., Karlin, R.J. and Alen, M.J. (2000) *Escherichia coli*: the best biological drinking water indicator for public health protection. J. Appl. Microbiol. 88, 106S–116S.
[71] Leclerc, H., Mossel, D.A.A., Edberg, S.C. and Struijk, C.B. (2001) Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annu. Rev. Microbiol. 55, 134–201.
[72] Gleeson, C. and Gray, N. (1997) The coliform index and waterborne disease, problems of microbial drinking water assessment. E&FN Spon, London.
[73] Robertson, J.B. and Edberg, S.C. (1997) Natural protection of spring and well drinking water against surface microbial contamination. I. Hydrogeological parameters. Crit. Rev. Microbiol. 23, 143–178.
[74] Newby, D.T., Pepper, I.L. and Maier, R.M. (2000) Microbial transport. In: Environmental Microbiology (Gerba, C.L., Maier, R.M. and Pepper, I.L., Eds.), pp. 147–176. Academic Press, London.
[75] Edberg, S.C., Leclerc, H. and Robertson, J. (1997) Natural protection of spring and well drinking water against surface microbial contamination. II. Indicators and monitoring parameters for parasites. Crit. Rev. Microbiol. 23, 179–206.
[76] Leclerc, H., Edberg, S.C., Pierzo, V. and Delattre, J.M. (2000) Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. J. Appl. Microbiol. 88, 5–21.
[77] Yates, M.V., Citek, R.W., Kamper, M.F. and Salazar, A.M. (1999) Detecting enteroviruses in water: comparing infectivity, molecular, and combination methods. Presented at Int. Symp. AWWA on waterborne pathogens, 29 August–1 September.
[78] Abbaszadegan, M., Stewart, P. and LeChevallier, M. (1999) A strategy for detection of viruses in groundwater by PCR. Appl. Environ. Microbiol. 65, 444–449.
[79] Beuret, C., Kohler, D. and Luthi, T. (2000) Norwalk-like virus sequences detected by reverse transcription-polymerase chain reaction in mineral waters imported into or bottled in Switzerland. J. Food Prot. 63, 1576–1582.
[80] Codex Alimentarius (1997) Draft revised standard for natural mineral waters. Alinorm 97/20, appendix II.
[81] Falkinham, J.O., Norton, C.D. and LeChevallier, M.W. (2001) Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. 67, 1225–1231.
[82] Covert, T.C., Rodgers, M.R., Reyes, A.L. and Stelma, G.N. (1999) Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. 65, 2492–2496.
[83] Atlas, R.M. (1999) *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol. 1, 283–293.
[84] Swanson, M.S. and Hammer, B.K. (2000) *Legionella pneumophila* pathogenesis: a fateful journey from *Amoebae* to macrophages. Annu. Rev. Microbiol. 54, 567–613.
[85] Bornstein, N., Marmet, D., Surgot, M., Nowicki, M., Arslan, A., Esteve, J. and Fleurette, J. (1989) Exposure to Legionellaceae at a hot spring spa: a prospective clinical and serological study. Epidem. Inf. 102, 31–36.
[86] Hardalo, C. and Edberg, S.C. (1997) *Pseudomonas aeruginosa*: assessment of risk from drinking water. Crit. Rev. Microbiol. 23, 47–75.