Microbiological characteristics of natural mineral water (*)

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Carbon dioxide-free mineral water, like any other natural spring-water, is never entirely free from bacteria; but it is always very low in bacteria. After the bottles are filled, in a few days or weeks, depending upon the environmental conditions, the few present bacteria increase markedly. Often, the number of bacteria is as high as 10,000 per ml, and even as high as 100,000 per ml. In this regard, a large number of quantitative analyses have already been carried out [1-7]. Quantitative analyses based on the various types of bacteria and on their specific properties, are presently still scarce in number [8, 9].

For the microbiological characterization of carbon dioxide-free mineral water, the specific microbiological and ecological conditions in this special substrate, must be chiefly considered. But since no analyses of mineral water exist that are oriented towards ecological factors, it will be necessary to use as a term of comparison the results relative to other waters low in nutrients, which exist in nature. Quite similar ecological conditions are found in free-flowing underground waters, in spring-water, in all oligotrophic waters and also in the sea waters that are extremely low in nutrients.

Comparable ecological conditions are also found in drinking water supplied by public plants, although drinking water, unlike natural mineral water, is a technologically processed product. Not even drinking water is always totally sterile; it is comparatively low in bacteria, but they are almost always present in an amount higher than in mineral water.

The known values indicative of the number of aerobic colonies in the order of magnitude of 20 to 100 per ml, are merely technological qualitative features for the qualitative control of the drinking water supply plant. Quite often, even if the quality is excellent at the time of supply, the drinking water that reaches the consumer's tap is bacteriologically of a much lower quality.

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As in the case of the bottles filled with mineral water, also in that of drinking water after a protracted stay in the pipes inevitably brings about a secondary increase in the number of bacteria present in it. In all the most important pipeline systems, both private and industrial, many pipe sections exist in which water stays for long periods [5, 10–12].

A particularly massive increase in the number of bacteria is repeatedly observed in internal water post–treatment plants, as in filters, phosphate feeders, ion–exchangers and pressure tanks [5, 13–16]. This secondary growth of bacteria in the pipeline system may even start from within the local distribution network and continue in the consumers' network [17–22]. A major role is certainly played by the general eutrophization of drinking water — that is a comparative enrichment in nitrate and phosphate — but chiefly of some trace amounts of organic substances [10, 23].

In the pharmaceutical industry, for a number of reasons, an additional regeneration of drinking water is already necessary [25]. In view of the deterioration of the bacteriological quality of drinking water that will be increasingly felt in the future, similar problems will occur in many other fields of the food industry. For this reason, the food microbiology must also deal with the problem to a larger extent. The carbon dioxide–free mineral water is only partly comparable, when bottled, with still drinking water, for the content in nutrients is remarkably lower. However, owing to the comparatively steady environmental conditions, this represents a good model for the microbiological processes — very much alike in principle — that take place in still drinking water, which is almost always subject to notable changes in environmental conditions.

In the microflora of carbon dioxide–free, bottled mineral water, there co–exist at all times two groups of bacteria, much different from one another both in origin and properties:

1. Allochthonous or contamination microflora;
2. Autochthonous microflora, or microflora proper of the substrate.

1. *Allochthonous or contamination microflora*

The allochthonous bacteria that get into the mineral water during bottling actually play a secondary role. We must however deal with them briefly in order that a clear–cut distinction may be made between them and the autochthonous flora proper of the substrate.

During the mineral water bottling, the following pollution sources enter the play: *a*) containers; *b*) caps, *c*) air environment, and *d*) bottling plants.

Containers. — Carbon dioxide–free mineral waters are nowadays almost always bottled into throwaway plastic bottles. The use of glass bottles is steadily losing ground. This is quite a satisfactory development from the sanitary angle, for the washed glass bottles in particular and their caps, represented the main pollution source [2, 4, 25–28].

As a rule, the plastic bottles are produced from granulate shortly before bottling. In the extrusion stage, temperatures as high as 230 °C are used for some minutes. In this way vegetative bacteria, conidia and, for the most part, also bacterial endospores, are killed. The experience acquired with dry sterilization by means of heat shows that such temperature–time conditions are not sufficient to kill endospores, or to make them inactive with a high degree of certainty [29]. But this point is still debated. Some authors [30–32] succeeded in proving a total inactivation of spores in the extrusion phase, whilst others were unsuccessful [33, 34]. It is quite certain that, as soon as they are produced, extruded plastic bottles are very low in bacteria and are contaminated only by a few bacillus endospores.

Caps. — As a rule, caps are also made in most cases of soft plastic materials, and are manufactured shortly before they are used. As in the case of bottles, they may at worst be contaminated by some endospores. When the bottles are conveyed to the open tank of the bottling equipment, as is most commune, a continuous secondary pollution may take place either through the air or through the parts of the machinery. In case of a mechanical breakdown, the caps should not be touched with the bare hands.

Air. — The main pollution of bottles and caps occurs via the environment air and via the compressed unfiltered air, when, shortly before bottling, air is forcibly let into the bottles and then let out of them. Such a pollution may be notably reduced by means of a sterile filtering of compressed air [35]. The polluting bacteria are chiefly the spores of bacilli, conidia and gram–positive bacteria most resistant to dry conditions, such as micrococci. Experience shows that the gram–negative bacteria are scarce in the comparatively humid air of bottling plants [35, 36].

Bottling plants. — The fast–action bottling heads must be always well lubricated. In the thin layer of oil, bacteria may survive and, under given conditions, they may even multiply. They may even get into the mineral water during bottling. A higher contamination risk exists if the equipment is to be repaired or if the heads to be changed are handled with unprotected hands.

Transitory contamination flora.

In the modern processes for the bottling of carbon dioxide-free mineral water in throwaway plastic containers, microbial contamination is much lower than before, when glass bottles were used. In some cases, pollution may be further reduced by adopting protection measures. Unquestionably, aseptic bottling would be technically feasible today, but for microbiological reasons the requests put forth at times in this direction appear to be unrealistic. On the one hand, the product to be bottled is not free from bacteria, on the other hand, the determining factor is not so much constituted by the absolute order of magnitude of the microbial contamination, as by the specific behaviour of the contamination flora in the bottled substrate.

The carbon–dioxide–free mineral water is a substrate which is much unfavourable to exacting microorganisms. It only contains trace amounts of organic matter in concentrations much lower than the lowest limits of the most sensible chemical verification methods, namely, more or less in the ppb range. In this substrate, a more or less notable percentage of polluting microflora, rapidly dies. A further percentage, either is slowly made inactive, or retains for a long time the ability of reproducing. But a reproduction proper very seldom occurs. Hence, it may be regarded as a prevailingly transitory polluting flora.

The number of exacting bacteria, killed or made inactive in substrates extremely poor in nutrients, is in relation to a large number of factors and may range between wide limits. As far as I could establish, no analyses of the carbon dioxide–free mineral water have been made available so far. But it has emerged from many works that a large number of bacteria are made 50 % inactive within 20 min, and 90 % inactive within 1 h, in distilled water, phosphate buffer solution, physiological solution of common salt, Ringer's solution, and also tap water. This does not apply merely to the various species of bacilli, for enterobacteriaceae and for many other bacteria, but also to *Pseudomonas fluorescens* and *aeruginosa* [37–42].

The cells of the bacteria that survive this environmental change, which is similar to a shock, are slowly made inactive and may be capable of reproducing even for a long time. Thorough studies have been carried out to try and clear up the debated question of the indicating value of *E. coli* and the coliforms, with regard to the fecal pollution of water. The results are partly contradictory. In most cases, however, it was found that also in the case of *Enterobacteriaceae* in natural waters a reduction takes place (more or less gradual according to the environmental conditions) in the number of the cells capable of reproducing, whilst no reproduction ever occurs [43–50].

Soil bacteria as well as *E. coli* may also reproduce in extremely diluted laboratory media [51–53]. The lowest limit–concentrations for growth for *E. coli* were measured at 0.28–0.5 mg organic matter /l [43, 54–56]. Zobell and Grant [58] have found growth of *E. coli*, *Staphylococcus citreus*, *Bacillus megatherium*, *Proteus vulgaris* and also of *Lactobacillus lactis* even in solutions with only 0.1 mg of glucose /l.

Similar experiments on mineral water have so far only been performed by Buttiaux and Boudier [8]. The AA. having let to stand *E. coli* and *Klebsiella aerogenes* in water passed through an autoclave or filtered and moderately mineralized, did not find any signs of reproduction, but only a slow reduction of the viable cells after 3 months at + 14 °C. Conversely, a surprising net increase was observed in highly mineralized water.

In some experiments that we carried out, personally after placing *E. coli*, *Pseudomonas aeruginosa*, *Salmonella*, staphylococci and strains of bacilli in an initial concentration of 10^4 cells/ml in untreated and bottled mineral water, we found no growth, during four weeks at + 20 °C, but only a slow decrease in the number of viable cells. Some given effects, antagonistic or competitive in respect of the autochthonous flora, probably enter the play.

Summing up, we must state that the polluting microflora that gets into the carbon dioxide–free mineral water during bottling, is prevalently transitory, i.e. no multiplication occurs as a rule but only a more or less gradual inactivation of pollutants.

Permanent contamination flora.

From the sanitary angle, the problems involving the bacteria that might be defined « permanent contamination microflora », are much more difficult to solve than those involving the bacteria of the transitory contamination microflora. The former are a specific group of gram–negative bacteria that may develop rapidly and abundantly in substrates extremely poor in nutrients. They are, so to say, « oligocarbo–tolerant ». Their most important representative is *Pseudomonas aeruginosa*. Such species of *Pseudomonas*, as, for instance, *Pseudomonas cepacia* (*multivorans*) and certainly also an entire group of strains of *Pseudomonas fluorescens*, also fall within this group. They usually originate from man and from animals and they are all clearly mesophilic. Therefore, they can develop at + 37 °C but also at higher temperatures, up to + 42 °C. Prevalently, however, they are not psychrotrophic.

The potentially pathogenic species of *Pseudomonas*, *aeruginosa* and *cepacia* (*multivorans*), are acquiring an increasing importance as dreaded pathogenic agents of secondary infections resistant to antibiotic drugs in the hospital sector. *Pseudomonas aeruginosa* is also likely to be the pathogenic agent of unspecific food intoxications. In recent years, a large number of analyses have shown that its reduced requirements and its marked ability of reproducing and surviving in any type of water low in nutrients, as well as its strong resistance to disinfectants, make possible its widespread use in all clinical sectors.

A massive increase of *Pseudomonas aeruginosa* and a protracted period of survival in a high number of cells, were proved: in distilled water [57–61], in deionized water [16] and in pure mineral salts solutions [61, 62]. The same also applies to *Pseudomonas cepacia* [63–66] which was found even in disinfectant solutions [64, 67, 68].

The strains of *Pseudomonas aeruginosa* and *cepacia* grown in distilled water present significant morphological and physiological differences in respect of similar cultures in conventional nutrient media [60, 66, 69]. In distilled water, cells are much smaller and unflagellated. With a number of cells higher than 10^7/ml, water is still perfectly clear. After the transfer into distilled water, without any « lag » phase, a rapid multiplication occurs. After the inoculation from standard laboratory cultures into distilled water, there takes place, instead, a decrease in the number of bacteria for some decimal potencies before reproduction begins. The cells from distilled water are liable to grow further up to $+ 12\,^{\circ}C$ or $+ 18\,^{\circ}C$. The cells from richer nutrients do not grow, instead. Furthermore, the distilled water cells clearly show a higher resistance to disinfectants.

These permanent pollutants « oligocarbo–tolerant » are clearly by far more dangerous, from the sanitary viewpoint, than the transitory contaminating flora, for they can presumably also increase in mineral water. It is thus necessary to adopt all the suitable measures to prevent a contamination, particularly of the bottling–plant resulting from the operators working bare–handed. In case of a shut-down of the plant, bacteria may enter the pipelines and multiply right in the terminals, in the tanks and in other places where water stays for a while. It is in these places that the running mineral water might undergo a constant contamination prior to bottling.

It is difficult to ascertain whether, and to what extent, such a danger actually exists in practice. At any rate, and as far as I know, the numberless bacteriological controls have never shown the presence of *Pseudomonas aeruginosa* or *cepacia* in bottled mineral water, in significant amounts. It is likely that the autochthonous reproducing bacterial flora produce an inhibition by competition with antagonistic effects. Contrary to untreated mineral water, distilled water and other types of water, in the clinical sector, are primarily sterile and after infection, almost always a pure culture of these *Pseudomonas* is present. It is quite possible, therefore, that the autochthonous bacterial flora in carbon dioxide–free mineral water, affords an effective protection against the reproduction of such permanent pollutants.

An effective bacteriological control for detecting this specific contaminating flora is already possible by counting the cultural colonies on a rich nutrient medium and by incubating at $+ 37\,^{\circ}C$. If the indicative value of « less than 1 colony/ml », recommended by the Scientific Commission of the

3rd International Symposium on Food Microbiology, held in Evian in 1960, is complied with, then an adequate security from the sanitary angle should be guaranteed.

2. *Autochthonous microflora, or microflora proper of the substrate*

The mineral water coming directly from the spring and immediately after bottling, is always very low in bacteria. The aerobic colonie count is in the order of magnitude of 1–2/ml [2], if the colonie counting methods are used, such as, for instance, pour plating with standard nutritive media and an incubation at 20° or + 22 °C or filtering by means of a membrane filter with pores 0.5 μm large and filter incubation, still on standard nutritive media. Using cultural media very low in nutrients, with surface culture procedures and longer periods of incubation, or using enrichment media low in nutrients in MPN procedures, also those bacteria are considered that are sensitive to higher substrate concentrations and to the heat shock in pour plating procedure. As a rule, the number of colonies is higher, it is in the range of 10 to 20 ml, and should correspond to the actual number of bacteria.

If membrane filters with pores 0.1 μm large are used, the number of colonies increases remarkably. Obviously, a large number of cells are smaller than 0.5 μm. In point of fact, the unusually small size of cells is a special feature of all autochthonous microflora. Cells are usually large only 1/3 to 1/4 of the normal bacterial cells, with a diameter comprised between 0.2 and 0.5 μm. They are irregularly coccoid and it is often difficult to recognize them at the microscope as bacterial cells. Even in the case of an intense increase in bottled water, the cells become only slightly larger, and thus even if the number of cells is very high no water clouding occurs. These so–called degenerated forms, that are however still capable of reproducing, are characteristic of many biotopes extremely low in nutrients in nature [70, 71]. In the long term, the mean number of cells capable of reproducing in continuously drawn spring water, is rather constant. In the meanwhile, however, longer or shorter oscillations may certainly occur, with bacterial numbers much lower or even higher and with the composition of species temporaneously changed to a marked extent.

Origin of autochthonous microflora.

The question repeatedly put forward as to the origin of bacteria in spring water, cannot be answered in a clear–cut way if experimental methods are used. According to all the microbiological–ecological experiences carried so far, it should be rather certain that this microflora multiplies directly in the water of the entire spring, and that it renews continuously. It seems therefore quite unlikely that the bacteria are dragged in a continuous flow from the surface of the soil, and that they are continuously carried forward through cracks, crevasses and clefts in the stone. This certainly occurs sporadically and results in a subsequent pollution of the spring water and nothing else.

The whole spring is an open flow–system with an inflow and outflow of water, whose rate of flow may be more or less variable depending upon the size and capacity of the entire spring. As a whole, a more or less uniform rate of flow should result. Bacteria may be in suspension in free water and adhere to solid surface [72, 73]. The cells of the bacteria suspended in water can meet their nutritional requirements almost exclusively through the uptake of dissolved lower molecular organic substances. A decomposition of the higher molecular substances, which is effective for the single cell, is possible, at the best, by means of enzymes bound to the cell surface wall and not by means of exoenzymes. Particularly the higher molecular substances are preferably absorbed at surfaces of all types. The surface flora, that usually does not adhere very strongly, is in a position to exploit to better advantage such an enzymatic decomposition, in view of the fact that the routes of diffusion of the low molecular scission products are very short. It is however possible to experimentally obtain, in water extremely low in nutrients, a significant speeding–up of bacterial growth by simply enlarging the internal surface of the aqueous body [54, 77]. The lower in nutrients a natural water, the greater will usually be the amount of attached bacteria in relation to those in free suspension [70]. The attached flora also prevails in underground water [78]. It is difficult to establish in which phase the greatest multiplication of bacteria takes place in underground springs. This is largely dependent upon the type of spring. The attached flora is certainly the most important in the majority of cases.

The heterogeneous open flow–systems present in nature are often compared, from the microbiological angle, to a continuous culture, such as, for instance, in chemostate. In the chemostate with a steady nutrient flow and an equal discharge, there rapidly occurs a flow balance (steady state), provided that all other conditions remain unchanged. All the cells are in the logarithm growth phase. Nowadays, experimental ecology successfully utilizes chemostate for ecology–oriented experimental models [79–84].

In natural waters, extremely low in nutrients, the metabolic rates of organic matter are exceedingly low, and do not allow direct measurements of the metabolic activities and of the production of bacterial substance. In the « steady state » experimental chemostate, even in very low nutrient concentrations, it is possible to determine the ration (irrespectively of the time factors) between cultural conditions and the growth characteristics of an organism. In this way, some strains of bacteria with exceedingly low specific rates of growth, usually unidentifiable, can be isolated and analyzed. Only in chemostate, it is possible to make a closer study of both growth and metabolism at submaximal rates of growth, as they are prevalently found in nature, in substrata low in nutrients and at the same time cool, also analyzing the mutual competition among such species [84].

In open natural systems certainly no microbiological flow balance exists. They do not in any way correspond to a chemostate. The growth of natural bacterial populations in such an open heterogeneous flow–system can take place, depending upon the local conditions, in part as a static culture and in part as a continuous culture. But it is certainly possible that in the very « natural spring » system, a flow balance of bacterial reproduction may be established at least temporarily. Quite often, notable fluctuation in the number of bacteria, with an initially high bacterial expulsion, are followed by periods almost entirely free from bacteria. In given occasions, the washing–out effects are similar to the processes occurring in a chemostate.

On the basis of the present, chiefly indirect experience, acquired in the field of experimental ecology, it is possible to assume with a certain degree of certainty that a true and significant multiplication of the autochthonous microflora takes place in the underground springs area.

Multiplication of the autochthonous microflora in the closed system.

In the water bottled directly from natural springs, low in nutrients, the environmental conditions for bacterial growth undergo, along with the limitation in volume, a drastic change, passing from an open flow system to a closed system. Shortly afterwards, a sizable multiplication of bacteria occurs. The natural mixed populations show the same regularity of growth as that of pure cultures in a static culture, with a lag, a logarithm and stationary phase. The one difference lies in the fact that in water very low in nutrients, a rapid decrease in the number of cells capable of reproducing occurs after the attainment of the stationary phase. Often, a further increase in the number of cells is attained in a second exponential phase. This may even occur more than once during a long period of stay. As it seems, the products of the autolysis and disgregation of the dead cells are utilized as nutrients for the new populations with a species composition that is almost always different.

Such a rapid multiplication of bacteria after bottling is typical of all nutrient–low waters. This was repeatedly confirmed by a large number of analyses, as, for example, in the case of river water, underground water, oligotrophic lakes water, sea water [5, 57, 85–88] and also in the case of drinking water [5, 7, 89], of deionized and distilled water [6, 90–93], and finally also in the case of carbon dioxide–free mineral water [6, 7, 8–28].

It is worth noting, however, that this rapid bacterial growth after bottling also occurs when all the other environmental conditions (temperature in particular) are maintained perfectly similar to those prevailing in the open system whence the water was taken [77, 86, 94]. This only applies, however, to water exceedingly low in nutrients. The internal surfaces seemingly allow a surface adsorption of the few organic matters present. This fact alone actually allows a better exploitation by the bacteria [54, 57, 95] which probably settle on the surfaces. According to the studies carried out by Barbesier [96] the inner wall of the plastic bottles looks quite rough under a microscopic examination. After a long period of time from bottling, crystal–clear and filamentous stratifications are found, that are irregular and with interposed bacterial colonies.

In bottled mineral water, two other growth–promoting factors become relevant. During bottling, water is more or less substantially enriched with oxygen. Furthermore, temperatures during the period of storage are usually much higher than at the spring.

The multiplication of bacteria in bottled water very low in nutrients, is a perfectly normal biological process. It may be prevented only through a sufficient water pasteurization or sterilization.

Geldreich and Clark [93] point out that in the absence of bacteria in any water unsterilized and low in nutrients, such as, for instance, in distilled water, the doubt always exists of the presence of toxic substances.

Bacterial species of the autochthonous microflora and their properties.

Well grounded analyses of the bacterial species of the autochtonous microflora in natural mineral waters, and of their specific physiological properties, are not yet much developed. This also applies, as it is obvious, to all other mineral waters low in nutrients. In the absence of more accurate data, only rather summary statements are possible.

In waters low in nutrients, gram–negative bacteria usually prevail. In deep underground water, microflora prevalently consists of *Achrobacter* and *Flavobacterium* species. There also exist gram–positive species of *Micrococcus* and *Nocardia* [78]. A microflora like this prevails in all springs, in streams and in oligothrophic lakes [97, 98]. Such *Pseudomonas* as *Pseudomonas fluorescens* in particular, are usually present only in the case of a stronger eutrophization [98]. Also found in natural mineral waters were *Achromobacter* and *Flavobacterium* species and also *Pseudomonas* and *Xeromonas* species [1, 8]. All these data must be viewed, however, with some reserve, either because a more accurate description of such species is not available, or because the descriptions at hand are so incomplete as to make it quite difficult to define also the genera according to the new taxonomic viewpoints [100].

As it results from personal experiences, in the autochthonous microflora of mineral waters, the gram–negative species prevail to an extent of over 60–70 %, with formation of yellow colonies belonging to the *Flavobacterium–Cytophaga* group. The comparative small number of species with no more than 3 or 4 different types in a changing relationship, was quite striking. Among the species that formed the yellow colonies, there were also gramvariable *Arthrobacter* species present. The remaining microflora chiefly consisted of *Alcaligenes* species, defined so far as *Achromobacter*. Typical *Pseudomonas* were found less frequently and always in a comparatively low amount.

The composition of species, after their cultivation on mediums low in nutrients, consisting of meat bouillon or mineral agar, that always gave the highest colonie count were determined. On the other culture media rich in nutrients, selectively species that may grow also at higher nutrient concentrations, are favoured. Many species of the autochthonous microflora proper, either show an insufficient growth when cultivated on that medium, or do not grow there at all. The often mentioned prevalence of *Pseudomonas* in bottled mineral water is thus only methodic, and in way reflects not the actual situation.

The major physiological feature of the autochthonous microflora is the marked oligocarbophilie, being the preference for solutions extremely low in nutrients. Oligocarbophilic bacteria may grow in mineral salts without any addition of organic matter. Such organic matters as those present in tap water, are in trace amounts and are quite sufficient. The minimal limit–concentrations for the organic matter are notably lower than the chemo–analytical verification limit [101].

It is repeatedly stated that during the period of bottling in plastic containers, the low molecular substances coming from the polymer, may be used by the bacteria present in mineral water as additional nutrients. Comparable experiments with tap water and mineral water however show no significant difference in the development of bacteria between bottles made of glass or plastic. The traces of organic substances, present in mineral water off the source are evidently sufficient for the growth of bacteria.

The definition of « autothrophic » [2] should be replaced by the word « oligocarbophilic ». No true autotrophie was found so far in any of these bacteria, and it is quite likely that it does not even exist. As far as we know now, all the bacteria of the autochthonous flora are clearly chemoorganotrophic. An optional chemolithotrophy, with H_2 and CO as a source of energy, cannot be however excluded — as it is known in the case of some *Pseudo-*

monas species. A heterotrophic fixing of CO_2 that, according to Kusnetsov and Romanenko [102], represents in water, a percentage of over 6 % of the bacterial biomass production, seems instead quite possible [103].

Nitrogen requirements are also clearly much reduced. The autochthonous microflora is simultaneously oligonitrophilic. Jannasch [71] succeeded in proving an evident growth of *Flavobacterium* aquatile at concentration lower than 1 γ of NH_4^+ nitrogen/l.

All the bacteria of the autochthonous microflora are aerobic, and in part facultative anaerobic. According to Clark and Burk [104] *Pseudomonas* and *Achromobacter* can grow even at oxygen concentrations lower than 0.5 %. Moreover, many species are capable of anaerobic respiration in the presence of nitrate or nitrite, whose oxygen acts as an electron acceptor. Owing to the comparatively low metabolism, only small amounts of oxygen are required. When bottled, water is sufficiently enriched with oxygen. A further oxygen diffusion through the plastic bottles, from the microbiological angle, should possibly occur only after several weeks.

Finally, all the bacteria of the autochthonous microflora are unquestionably psychrophilic (facultative psychrophilic). The minimal temperatures for a multiplication are of 0 °C. Such maximum temperatures as 25–30 °C are seldom exceeded. The optimum of growth is usually found at + 20 °C. In the case of low temperatures at the spring, multiplication is by far slower. Almost always, higher temperatures are used after bottling. This is sufficient to promote bacterial growth to a large extent.

Of major importance for the quantitative determination of the whole autochthonous microflora, is the correct choice of the culture and media conditions, in keeping with the specific environmental requirements. Already at the counting of bacteria in drinking water, the different culture media may cause considerable deviations both in the number of colonies and in the range of species [14, 105, 106]. For the oligocarbophilic microflora determination, culture media low in nutrients must be used instead. It is not yet clear the extent to which bacteria are obligate or facultative oligocarbophilic. *Pseudomonas* are certainly facultative oligocarbophilic for the most part. The number of colonies essentially higher on media low in nutrients, point to the fact that a high percentage of flora is obligate oligocarbophilic. These species, in the subsequent culture in conventional media, are very unstable and can be still cultivated successfully only on adequately poor media.

Summing up, we must state that the autochthonous microflora in mineral water, as well as in all other waters very poor in nutrients, existing in nature, is markedly oligocarbophilic and psychrotrophic. We presently know very little of the bacterial species and of their physiological properties. The autochthonous flora may multiply slowly and renew constantly in the open flow–

systems of natural springs. After spring water is bottled, there occurs in the container a more or less rapid bacterial multiplication, as in static cultures. This is mainly due to the increase of the inside surface, and also to the oxygen enrichment and to the higher temperatures during the storage period.

We must expressly point out, by way of conclusion, that the increased multiplication of bacteria after bottling of water very low in nutrients, is an altogether normal biological process. The indicative or threshold values of the number of aerobic colonies at a 20 °C incubation for an untreated water, are absolutely meaningless from the biological viewpoint [106]. Objectively speaking, only 2 possibilities are left: either the renunciation to any indicative value as to the number of aerobic colonies st 20 °C, or the need for a number of bacteria equal to 0 for each bottle. The latter alternative is possible only through pasteurization or sterilization. But in this case the product is no longer in its natural state. From the sanitary angle, of importance is the constant bacteriological control of the allochthonous polluting flora, and chiefly of the transitory polluting flora, through the determination of the number of aerobic colonies at a 37 °C incubation.

Summary. — Natural, non-carbonated mineral water is, like every other natural water from a spring, never sterile. However, the microbial level is always very low. But after its bottling, the level rises rapidly and numbers of more than 10,000 to 100,000/ml can be reached. In principle 2 groups of bacteria of very different origin and properties can be found in the microbial flora of the bottled, non-carbonated mineral water.

Allochthonous bacteria will get into the water by contamination from the containers, closures, air or the bottling machines. They are mostly transitory as they cannot grow in a substrate with an extremely low nutritive level and die off more or less rapidly.

From the hygienic point of view the permanently contaminating flora with *Pseudomonas aeruginosa* as main representative is more serious. These special gram-negative bacteria are oligocarbotolerant and can therefore multiply in the mineral water of extremely low nutrient level after a certain adaptation. Their effective bacteriological control is possible by colony counting with incubation at + 37 °C but only just after bottling.

The autochthonous microbial flora consists of psychrotrophic and of distinctly oligocarbophilic, mainly gram-negative bacteria such as *Achromobacter*, *Flavobacteria*, *Pseudomonas* as well as gram-positive *Arthrobacter*-species. According to indirect experiences, this autochthonous microbial flora must be growing in the open system of the underground source and renew itself constantly.

The bottling of the natural spring water implies a drastic environmental change from this open system into a closed one. Then the bacteria start

multiplying more or less rapidly like in a batch culture. Main reason for this is the extension of the inners surface of the system.

The multiplication of bacteria after bottling of a mineral water of extremely low nutrient level therefore is an entirely normal biological process. For this reason, limits of the aerobic colony count at + 20 °C incubation for natural mineral water seem not to be justified.

Résumé *Les caractéristiques microbiologiques de l'eau minérale naturelle, non gazeuses*). — L'eau minérale non gazeuse n'est, comme toutes les autres eaux de sources naturelles, jamais tout à fait stérile, mais toujours extrèmement pauvre en germes. Après l'embouteillage un accroissement significatif des microorganismes présents dans l'eau minérale est constaté et s'élève à un nombre atteignant 10.000 à 100.000 germes/ml.

En principe, les groupes de microorganismes de la flore microbielle dans l'eau minérale non gazeuse peuvent être d'origine et de caractère très divers.

Les microbes allochthones entrent dans l'eau par contamination par les bouteilles, par les capsules, l'air ou les installations d'embouteillage. La plupart des microbes ne se présentent là que de façon transitoire, car ils ne se multiplient pas dans des substrats pauvres en substances nutritives, et meurent assez rapidement.

Ce qui est plus inquétant du point de vue hygiènique est la contamination permanente provoquée par *Pseudomonas aeruginosa*. Ces bactéries gram-négatives sont oligocarbotolérantes et peuvent se multiplier même dans l'eau extrèmement pauvre en substances nutritives, après adaptation. Un contrôle bactériologique effectif est possible par la détermination du nombre de colonies à + 37 °C immédiatement après l'embouteillage.

La flore bactérielle autochthone est composée de bactéries psychrotrophes et bactéries oligocarbophiles et gram-négatives comme les groupes de *Achromobacter*, *Flavobacterium* et *Pseudomonas* comme également les groupes de *Arthrobacter*. Après les expériences receuillies de façon indirecte, la flore microbielle autochthone peut déja se multiplier lentement dans le système ouvert des sources souterraines et se renouveler constamment. Après l'embouteillage de l'eau de la source les conditions du milieu pour l'accroissement des bactéries se modifient totalement d'un système ouvert à un système restant. Un accroissement de bactéries plus ou moins rapide commence, c'est-à-dire comme dans une culture statique.

La raison principale en est l'accroissement des surfaces internes.

L'accroissement plus intensive après l'embouteillage de l'eau minérale pauvre en substances nutritives est donc un processus normal du point de vue biologique. Les valeurs limites du nombre de colonies aérobies à + 20 °C ne sont donc pas biologiquement significatives pour l'eau minérale naturelle.

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