Assessing the microbiota of waters from portuguese dams

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Microbiology

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“If we knew what it was we were doing,
it would not be called research, would it?”
— Albert Einstein

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Abstract

Since the dawn of the simplest forms of life on Earth until the most complex ecosystems that can be recognised nowadays, all of them, strictly, depend on water. Sustainable systems for drinking water supplying are challengeable tasks; to assure the ellegibility of those waters for drinking, microbiological controls are needed.

In this preliminary work, 26 samples of water (21 portuguese dams) were collected from September 2014 to March 2015. The experimental plan included the enumeration of total cultivable microorganisms, coliform microorganisms, *E. coli*, and *Enterococcus* spp., using methodologies based on national and international standard procedures, to evaluate dams' water characteristics. Due to their potential hazardous activity and as an indicator of the water conditions, and its trophic condition, a methodology to detect cyanobacteria and green algae was drawn up and applied in the present work. Enteric bacteriophages were also searched.

Results support the idea that physical stratification of the water column is a factor influencing the microbial burden of these surface waters. It also showed that the frequency of potentially hazardous cyanobacteria is high, stressing the need for having always it in consideration as a parameter. Attending to official standards in use, the results allow different classifications: 10 samples showed contaminations compatible with the A1 ranking, 16 quality A2 and none had quality A3.

Each indicator has its own ecological behaviour and specific responses to environmental stressing factors. To safeguard the adequate "water quality", it is important to use multiple fecal indicators, avoiding possible hazards and health risk.

Keyword: dams´ waters, indicators, microbiota, cyanobacteria.

Resumo

Desde que surgiram as formas mais elementares de vida na Terra até aos ecossistemas mais complexos da atualidade, tudo depende estritamente da água. Garantir a existência de sistemas sustentáveis de abastecimento de água potável são tarefas cada vez mais desafiadoras; para assegurar a potabilidade dessas águas, são necessárias diversas monitorizações incluindo as microbiológicas.

Neste trabalho preliminar, foram recolhidas 26 amostras de água (21 barragens portuguesas) entre setembro de 2014 e março de 2015. O procedimento experimental consistiu na contagem de microrganismos cultiváveis aeróbicos totais, coliformes, *E. coli* e *Enterococcus* spp. utilizando-se metodologias baseadas em padrões nacionais e internacionais, tendo em vista a caracterização da microbiota das águas de barragens. Devido aos potenciais efeitos adversos associados à presença de fitoplâncton, foi aplicada uma metodologia para detetar cianobactérias e micro algas. Também se pesquisaram bacteriófagos entéricos.

Os resultados apoiam a ideia de que a estratificação física da coluna de água influencia a carga microbiana destas águas superficiais. Também foi evidente que a frequência de cianobactérias potencialmente perigosas é elevada salientando-se a necessidade de ter estes agentes em consideração como um parâmetro qualitivo. Atendendo aos valores paramétricos oficiais em uso, os resultados permitiram diferentes classificações: 10 amostras apresentaram contaminações compatíveis com a classificação A1, 16 qualidade A2 e nenhuma qualidade A3.

Cada indicador tem o seu próprio comportamento ecológico e respostas específicas a fatores de estresse ambiental. Para garantir a adequada "qualidade da água", é importante o uso simultâneo de vários indicadores, tendo sempre em perspetiva a salvaguarda da saúde dos respetivos utilizadores.

Palavras-chave: água de barragens, indicadores, microbiota, cianobactérias.

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List of Abbreviations

Abbreviation	Description
BG	Blue green
CFU	Colonies forming units
EPA	Environmental Protection Agency
FC	Fecal coliforms
FLA	Free-living amoebae
HAdVs	Human adenoviruses
HAV	Hepatitis A virus
Log_{10}	Logarithm base 10
MDGs	Millennium Development Goals
MF	Membrane Filtration
MPN	Most Probable Number
PCR	Polymerase Chain Reaction
TBA	Tryptone Broth agar
TC	Total coliforms
U.S.	United States
UN	United Nations
UNESCO	United Nations, Educational, Scientific and Cultural Organization
UNICEF	United Nations Children's Fund
UV	Ultra violet
WHO	World Health Organization

Chapter I – Dam water and Microbiology

1. Introduction

Since the dawn of the simplest forms of life on Earth until the most complex ecosystems that can be recognised nowadays, all forms of life strictly depended on water and had emerged from it - Life is a gift of the water. It is essential to all living beings (in quantity and quality), an indispensable nutrient for them, and to the development of all communities in the world. All living organisms have water in their composition because it is the basal matrix in which most of the metabolic reaction take place, sustaining growth, development and evolution [1].

The abundance of liquid water is the most significant difference between Earth (causes the blue color) and other planets of the solar system. Without it, the natural equilibrium would be disrupted and the ecosystems collapsed, the planet Earth would be a sterile rock - it builds the world as we know it, occupying two thirds of it. Despite this, from of all the water on the planet, only 2.5% of it is freshwater, having the possibility to be used for consumption (0.3% of this is rivers and lakes) (Figure 1.1).

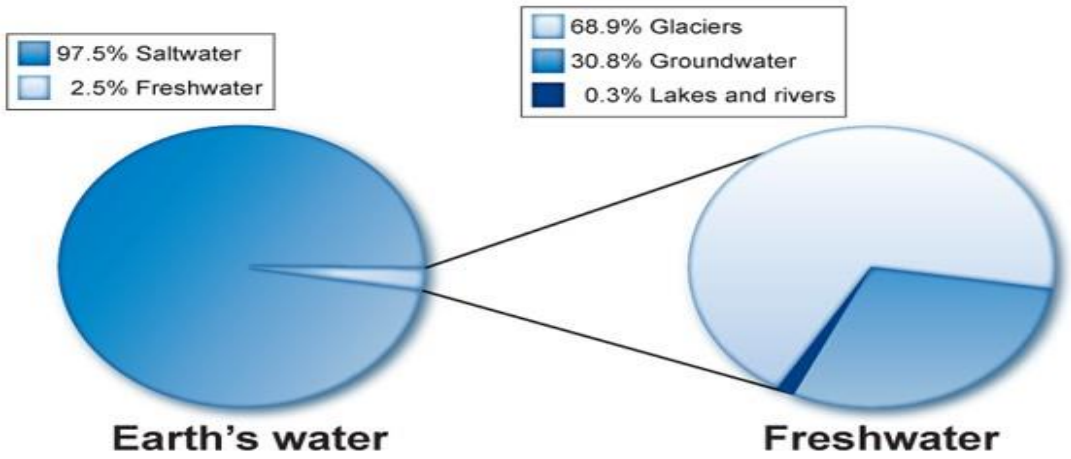


Figure 1. 1 - Relative frequency of Earth water and freshwater (adapted from reference [2])

This reduced availability means that not everyone gets the same possibility to access it in a regular way, or even when close to a source, may be polluted or unfitted to be consumed [3]. One recognized source of pollution is wastewater, it represents a risk of contamination if drained close to a non-polluted freshwater collection compromising the safe access to water [4].

Other limitation to the availability of water since the beginning of history is poverty. It has been a major barrier to have access to clean drinking water and sanitation in many parts of the world [5]. In 2006, a report from the World Health Organization (WHO) reveal that 1 billion of the world population living in poverty does not had access to safe drinking water and almost 2 million people died every year (majority of whom are children) from water-related diseases (e.g. diarrhea, dengue fever and typhoid). The most common cause of death in children is diarrhea, killing 1.5 million children each year [6]. However, it is important to refer that water shortages and pollution have consequences in both industrialized and in developing countries.

All of these advantages and limitations, makes the potable water one of the most precious resources, a reason to be a main topic in numerous discussions concerning the sustainability of mankind future, reflecting in how humanity may survive and what must change in order to preserve it adequately.

The future of mankind is uncertain but is know that the most prosperous societies of the antiquity were built around water (e.g. the Egyptian civilization was organized around the Nile River) and water has been a decisive factor in their expansion or development. As populations grew in these societies, the water was becoming a feature increasingly exploited and during this process, the water needs were always increasing. To support those growing needs, more sophisticated systems to obtain the water, save it (reservoirs) and distribute it (aqueduct) had been applied.

The pressure on the already scarce resources will lead to a generalized demand and a bigger dependence of the administrations to monitor and to detain it in a safe way. Keeping the current levels of exploitation, to sustain the increasing needs, and the exponential growth of the world population. This crescent rise in population, and the extensive effort to keep it, has caused a progressively higher consumption (comparing to previous years), and it triple in the last 50 years.

A large fraction of humanity has scarce access to water (defined as water-stress) [7]. Without enough water, a restriction to the maximum potential a user can achieve, conduces to the loss of an essential step in human development [8].

In 2005, a global initiative - the “International Decade for Action: water for life” - started as an effort to accomplish the Millennium Development Goals (MDGs). Improvement of the access to potable water, reduce the number of persons below the water-stress threshold and improve their life conditions. It was decided to reduce by half the proportion of the world´s population without sustainable access to safe drinking water by 2015 [9]. This target was accomplished in 2010, and in 2012, already 89% of the world’s populations had access to an improved source [10].

Another document published by the United Nations and reporting information’s from 94 countries and 23 aid agencies, revealed that 1.8 billion people used a source of drinking water that is contaminated, but 2 billion people have gained access to clean water (being hard to judge the quality of drinking water). Thanks to this, the number of children dying of diarrhoeal disease has fallen from 1.5 million in 1990 to around 600.000 in 2012 [11].

In 2014, the UN issued a list of 17 draft sustainable goals, in which there were seven targets addressed to water. Two of them aim to guarantee the universal access to safe and affordable drinking water [12]. With these results and following the achieved advances, the “Sustainable Development Goals” replaced the 15-year-old “Millennium Development Goals (MDGs)”.

There are still several obstacles for quickly solving the current problems. In an article published in 2006, by Moe and Rheingans, it was pointed five major challenges as essential to provide safe water and sanitation on a global basis:

(1) contamination of water in distribution systems,

(2) growing freshwater water scarcity and the potential for water reuse and conservation,

(3) implementing innovative low-cost sanitation systems,

(4) providing sustainable water supplies and sanitation for mega cities,

(5) reducing global and regional disparities in the access to water and sanitation and developing financially sustainable water and sanitation services [13].

Some changes depend on good policies. Since July 2010, water and sanitation were considered a global public good by the General Assembly of the United Nations. Its access is a responsibility of the public governance, which should ensure and guarantee its quality. It was sent a resolution formally recognizing water and sanitation as basic human rights [8].

Similarly, in 2014, the European Council stated that water must be protected and well preserved, dispensing a special attention to surface waters [14]. A renewable source with a limited capacity to recover from the dangerous and irresponsible human behaviour or natural environmental disasters [15]. The supply of fresh water is ranked in the third place of the threats of greatest concern to the planet on a list elaborated by more than 700 business, government, and non-profitable institutions who responded to the WHO - based think tank's annual survey [16].

Systematic microbial examinations of surface waters are an extensive process worldwide put in place to ensure the safety of waters when used for different purposes.

The characterization of the water microbiota is the central objective of this work. Studding it, and the consequences of its presence, is an essential step to guarantee the safety of water and to contribute to a better understanding of its social significance.

1.1 Dams - History and Importance

Dams' construction is an old resource especially for irrigation and reserve of water. In the last century it become used to generate electric energy. Nowadays, a river with its spontaneous flow course is rare, attending that the exploration of these natural resources is evolving and intensifying every day.

Dams can promote economic development of the neighbourhoods compensating, at some level, the perturbations that are made in the natural landscape (Figure 1.2). Depending on a costly initial investment, transferring of populations and studies to evaluate the environmental impact. The dams built on rivers with international courses may disrupt the regularity of the water flows affecting land and populations positioned downstream. It makes the dam construction decision, not only a national issue, but also a matter that forces to an international management [18].

Since the first hydroelectric power plant on the Fox River in Appleton, Wisconsin (1882), it proved possible to generate electric energy from water force fall - an ecologic and renewable energy source. The utilization of the river waters as a source to produce energy started in Portugal in the end of the XIX century (the first plants were destined to regional business and to sustain weaving industries). The construction and improvement of dams has been constantly, supported by appropriate legislation.

Using the mechanical energy of water flow is possible to produce electric energy able to maintain the work of factories, or, after conduction, to public or private illumination of cities where replaces less renewable and polluted sources. This resource is renewable due to the water cycle, making it always available to produce energy and, depending on the dimension of the dam, it can play a significant role in the electrical production of a country [19]; for the moment, the energy potential of the Portuguese rivers is close to it maximal productivity [20].

Aligning this with the necessity of holding water in a reservoir for other uses like: irrigation, nautical activities, leisure and incentive to the local tourism, a dam became a symbol of human progress.

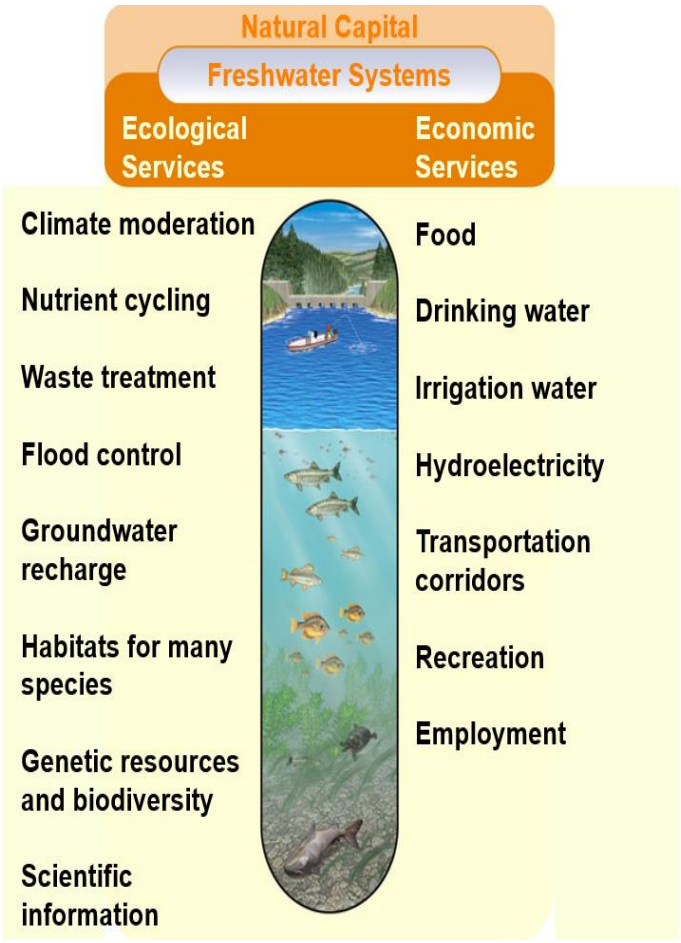


Figure 1. 2 - Freshwater ecological and economic services (adapted from reference [17])

One of the main roles of a dam is to offer a more manageable source or a reserve of water for consumption. It is a solution of humans to satisfy a primary necessity. When water is accumulated by this way, it forms an artificial pond. In regions where the water supply is a scarce resource, a reserve can be a huge advantage, allowing a constant flow of water, even in times of global warming, water remains one indispensable resource to sustain all life forms and populations.

Water naturally originated from the rain or used in an irrigation system is the key for crops growth. Irrigation is a very relevant way to satisfy the growing food demand of consumer's around the world, and water consumption is expect to keep growing [18]. Agriculture irrigation is the activity that uses the largest amount of water, representing almost 70% of total water withdrawals (industry 20% and domestic purpose 10%), being the main factor in the consumption future growth. It is also the most important factor in the water utilization imminent growth [9]. At least 20% of total arable cropland is under irrigation, producing about 40% of the global harvested crops, being a fundamental tool to ensure enough food supply in the future [21].

Some leisure activities are also largely dependent of the fresh water stocks and influence its use (sanitary uses, consumption, recreation and welfare). Public spaces (gardens, acclimations, golf camps, aquarium) also depend largely of water resources [22].

Many other uses of water could be refer but those previously referred show the diversity of applications assigned to water.

1.2. Water analysis

To achieve all the possible uses of water many characteristics need to be respect: its salts, chemicals, physical, organic, microbes, aquatic plants and animals. Several studies evaluated the possible influences of its characteristics in the fauna, flora and microbiota. Including those that arrive with the dam construction, disturbing normal cycles of living organisms, like migration, nesting, refuge, or places for feeding the animals.

Water is so critical to life that scientists and risk assessors constantly try to understand how human can be vulnerable to microbial, chemical or physical threats present in the drinking water supplying systems or in recreational waters, and how to avoid those exposure. It is important to emphasized that this steady water allows accumulations of organic and inorganic pollutants and the growth of many organisms, including hazardous, due to the natural exposure to the air (winds), soils and liquid effluents (sometimes with sporadic discharges).

Specific biogeographical regions of earth, where ecological communities are present (co-exist), under specific ecological conditions are named “biome” [23]. Within a biome, like that of a dam, artificially built, there are local factors influencing the survival of the different species. The structure of lentic water, (lake) ecosystems created by the construction of a dam is rather complex, subject to the dynamics that result from the geographic location of the dam and the intrinsic zonation of the water column. This zonation is determinant of microbiota distribution in those waters (Figure 1.3). The irregularity in microbial distribution is a feature that influences the results of microbial analyses. Even the direct human activity can pollute it, when swimming or practicing nautical activities on these water collections [24].

Microbial microrganisms in the benthic zone (lakefloor-hosted) are distinct from planktonic (free floating microrganisms), due to the water depth, sediment depth, and by energy availability (deposited organic matter) [24]. The littoral zone, surrounding the surface water body receive directly the sun light. It has plankton (both phytoplankton and zooplankton), small microorganisms that are the base level of the food chain. Without them no organisms could survive, humankind neither [25].

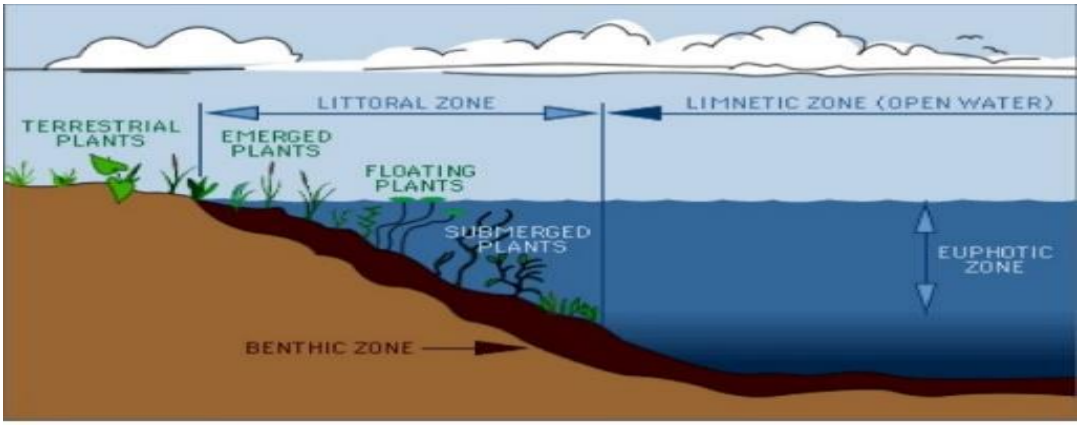


Figure 1. 3 - Example of the zonation in a lentic system (adapted from reference [26])

The reservoirs created in a dam are efficient in trapping sediments (70 - 90 per cent of the sediment volume delivered by the water flow) and is thought that 46 per cent of the water in the 108 most important rivers of the world, before arriving the sea, goes by a reservoir. This has an economic impact, because limits the mean life-time of a dam (is full with sediments) and promotes microbial

perturbations. This way, nutrients and contaminants are accumulated and may be remobilized, contributing to the primary production in lentic environments for bacteria, other micro-organisms, invertebrates and fish (there is also a minor production by algae and rooted vegetation) [27].

Humans influence the water quality, and the impact of water on human well-being, has been profusely described. Romans were the first people that had a profound cultural relationship with the welfare provided by the water, using thermal springs. In these thermal baths, comfort took such a social relevance that those leisure facilities became a place of social gathering in which the major policy decisions took place. Based on Roman experience, many people start building villages, towns and cities close to relevant water sources, e.g. on the bank of lakes, rivers or bays. This physical proximity of these human groups and water was intended for the best profit [28].

However, water has not always been a source of well-being, sometimes it has also been the source of disease. The impact of the inevitable human fecal excretion into closed water collections, transformed the solution in a nightmare. Water constitutes a source of dangers in this scenario.

John Snow was the first time physician that related scientifically (epidemiologically) a water source with a disease and managed the specific risk. During a cholera outbreak in London, in 1854, a drinking-water pump was recognized as the source of the agent. It had been possible to stop the outbreak of cholera removing the handle of the pump [4]. After that, microbes have been described, assessed, characterized and its management became a tool for control. Among those tools, available, analytical procedures were referred as the most reliable step to be assertive. Plate counting is the routinely oldest method developed by Robert Koch (1880) to enumerate microorganisms in water. The methodology was recognized in 1895 and in 1916, Breed e Dotherer formalized the procedure for this method [29].

Surveillance that was developed depends not only on biological parameters, but also on the evaluation of a set of physical and chemicals agents. They are chosen by their pertinence and relevance for the typification of the water quality. Many analytical procedures may be adopted having in mind the goal that must be assessed. The efficiency of this control is dependent of a schedule that must be structured attending to rigorous risk assessments. As a source of water for consumption, dams are permanently monitored and its water is treated, adding value to this precious good.

To schedule the hazards, systematic questions must be put in perspective: What are the living beings present in the water (including microbes)? How do they affect water characteristics? What are its identity, frequency and quantity of the pathogenic agents?

In a eutrophic dam, microbiota can grow in different proportions due to fact that the steady water provides the eugenic ecological conditions that favor specific microbial groups. Conditions like temperature, pH, salinity and the presence of nutrients, further improve the chances of bacterial survival and growth. Performing a quick search in the literature is possible to notice a vast number of articles describing the microbiota of water and its possible effect in humans (bacteria, virus, Fungi, Monera, and Chromista).

As previously indicated, diverse species of organisms growth in water environment. Most of the aquatic Plant, Animals or Microbes are generally characterized but some may been not yet identified: it is admitted that only a small part of the all spectre of microorganisms are routinely sought, among several that are search [30]. A minor number of these microorganisms had been incriminated in cases of animal or human deaths and diseases in living beings. None of these exclude the possibility of a

water exhibiting perfect conditions of color, transparency, taste or smell, may be responsible for health risks. Not only due to hazardous microorganisms, but also chemical hazards which effects are, sometimes, detected only in a time very distant from the exposure (causation).

An essential goal to provide safe drinking water is that it must be essentially free of pathogenic or potentially pathogenic microorganisms.

Microbial characteristics of water, intended for direct or indirect (ingredient) consumption, or with the potential to be found in contact with humans, are strictly regulated by legal frameworks in developed regions of the globe. Water suppliers and laboratory procedures follow normalized procedures, susceptible to be formally certified by regulatory bodies, official or private [31].

Whenever waters intended for consumption does not comply with the parametric limits stated by legal frames, many management procedures may be adopted. Physical and chemical biocidal treatments have been proved efficient for control “non conform waters”. Thermal treatments (boiling) were the first to be used in order to prevent *Vibrio cholerae* transmission. After that, many other have been developed like, ultra-violet light (UV), filtration, and especially chemical biocidal treatments. Chemical treatments have a smaller spectrum of action, although more practicable. Chlorination of drinking water was first promoted in the U.S. in 1908, and still today it is in use as a common inhibitory treatment for bacteria and viruses. However, some waterborne protozoa are resistant, requiring physical inhibitory treatments (filtration, UV or heating) [32].

Nowadays, there are countless procedures allowing the management of the hazards that may be found in drinking water. Some of those risk management procedures are stated officially and controlled by competent authorities.

1.3. Health risks assessment

Worldwide increasing demand for safe water is a natural consequence of the demographic evolution, since human population is growing permanently, like their necessities. To obtain enough potable water is a very stressing challenge, because to be potable it needs to be safe. Not all the natural surface waters present health risks, but they are very rare; that is the case of some protected natural springs used as thermal or mineral waters with fitness benefits. Microbial problems begin when surface waters suffer extrinsic contamination with chemical or microbial hazards, able to compromise its eligibility for consumption (Table 1.1) [33].

Prior to the early 1900's, waterborne disease was one of the most common causes of premature death due to the high number of pathogens agents’ vehicle by water. Chronic diseases started to be more observed in the 1990`s, with an evidence that microbes were responsible after human exposure to contaminated water. Even for diseases for which water does not seem to be evidently responsible: Gastric cancer, linked to *Helicobacter* spp., poliomyelitis, or Diabetes linked to Coxsakie B4 virus [34].

Infectious disease (epidemics, pandemic, endemic) linked to water had been referred in the ancient Greece and Egypt: epidemics of smallpox, leprosy, tuberculosis, and diphtheria - influencing politics, commerce and culture [35]. The number of waterborne infectious diseases outbreaks still reported around the world shows that pathogen agents in the water are still a very serious problem owing to the severity and frequency of those illnesses. The more common diseases are cholera, typhoid

fever, paratyphoid fever, infectious Hepatitis A, leptospirosis, viral gastroenteritis, cryptosporidiosis, amebiasis and bacillary dysentery [36]. To fully understand the role of water in diseases like legionellosis, bubonic plague, malaria or schistosomosis can be really complex. Modern processes of "risk assessment" are increasingly more holistic and have comprehensive approaches, specially due to the availability of accumulated data from re-emerging drinking water-related infections (which includes typhoide, cholera, shigellosis, rotavirosis, norovirosis) [37].

Table 1. 1 - Important waterborne pathogens in water supplies (WHO 2004)

Pathogen	**Infectious dose**	**Persistence in water supplies**	**Resistance to chlorine**	**Relative infectivity**	**Important animal source**
Campylobacter jejuni, C.coli	Low	Moderate	Low	Moderate	Yes
E. coli 0157:H7	Low	Moderate	Low	High	Yes
Enterovirus	Low	Long	Moderate	High	No
Cryptosporidium	Low	Long	High	High	Yes
Giardia intestinalis	Low	Moderate	High	High	Yes
Norovirus	Low	Long	Moderate	High	Potentially

For several researchers, the estimations of the total worldwide burden of waterborne disease is an objective. The used of figures, based on reported outbreaks, are believed to underestimate these problems because a significant proportion of waterborne illness is undetected by the surveillance and reporting systems [38]. This may be justify by the fact that some clinical signs are generally mild, lasting for short time and because only a small part of people uses the primary health care services (and not all of them are examined to detect the specific organism) [39]. In the U.S.A. (2002) it was believed that 560 000 people may suffer from a moderate to severe infections originated in water and 7.1 million suffer from a mild to moderate waterborne infection each year [34]. Some outbreaks (e.g. cholera) started in world regions where the population does not access safe drinking water; water becomes the principal responsible for transmissible diseases. Potential pathogen microorganisms (including those of human origin), are able to survive in water for different periods and some of them may multiply in the aquatic environment. This lapse of time can be sufficient to contaminate the water source, enhancing health risks (to those who are exposed through drinking water) and very significant economic losses. Indeed, some calculations estimate waterborne diseases for one-third of the total intestinal infections worldwide, while questions related to sanitation and hygiene were accountable for 40% of all deaths, and 7% of the total disease burden worldwide [34].

The five major groups of infectious agents transmitted by water are: bacteria, viruses, fungi, protozoa, and helminths. A brief review of the general characteristics of each of the agents belonging to these groups will be referred later.

Preventive measures against these microorganisms may have an almost null effect if the supply or distribution systems are deficient. In a public supply system of potable water, deterioration of pipes,

poor maintenance of water treatment and integrity of the distribution systems are associated to waterborne diseases in modern societies. Internal biofilms in pipes may also play a relevant role, protecting viral and protozoan pathogens from chemical biocides [40]. In those regions (and where sanitary treatments are absent), the population may suffer of more frequent clinical sign of intestinal infections due to the exposure to pathogens with high morbidity rates like *Vibrio cholerae*, Hepatitis A virus, Rotavirus, Norovirus and different pathotypes of *Escherichia coli* [34].

It should be notice that all this knowledge concerning the microorganisms present in water and their life cycle allowed to put in place management strategies aiming the reduction of diseases occurrence. The WHO estimates that improvements in the water quality could reduce the worldwide burden of disease by 10% percent [41].

Specific guidelines state that " water intended for human consumption shall be wholesome and clean if it: is free from any microorganisms and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health" [42].

The specific quality of drinking water is a mandatory characteristic. The Portuguese legal frameworks focused in its composition (physical and mineral) and in routine surveillance of microbial contaminations. As outlined in the 1st article of the portuguese legislation, Decret-Law n.º 306/2007 of August 27 - "aiming to protect the human health from adverse effects coming from eventual contaminations of water, and ensure an universal access to safe, clean and universal balanced water" [43].

Several international and national organizations, along the recent years, explore the value of water and its different uses, but a more efficient global coordination is needed (Table 1.2).

Table 1. 2 - Examples of contribute from organizations in relation to the improvement of water access and quality

Organizations	Contributes
WHO	Extend review about water resources uses in the planet and its influence in future life, recommending guidelines and objectives to preserve fresh water and, indirectly, the public health.
UNICEF	Has been working on more than 100 countries around the world improving access to safe water and sanitation and promoting hygiene awareness [44].
EPA	Promotes studies concerning pathogens and microbiological contaminants, *Aeromonas* spp., Cyanobacteria, Virus, *Helicobacter pylori* , elaborating protocols and guidelines, and advices for water treatments (e.g. *Giardia lamblia*, Virus, *Legionella* spp.) [45].

1.4. Microbiology of dams' waters

The artificial lakes that are created with the construction of dams, have an ecological organization that is similar to natural lakes. The waters present physical and chemical characteristics that are determined by: the geological structures of the soils, latitude, longitude, altitude of its location and by the frequency of pluvial inflows depend of raining regimes. The column of water, in ecological terms, may be organized in many ecosystems colonized by different species of organism (animals, plants and microbes).

The water body may present longitudinal and vertical stratifications concerning ecological organization, depending on the length of the river that feeds the hydrographic basin. In the body of the dams' water two major zones may be considered; the limnetic (central and deep) and limic (shore or littoral of the artificial lake). The limnetic zone is also stratified in terms of temperature, pH and luminosity, depending of the profundity of the water column. In this zone, the physical characteristics of waters are quite stable, without mixing with the fresh inflows.

The entire body of water is built on a bottom (benthos) whose sedimentary structure and topography gives rise to multiple ecosystems. Biotic and anthropogenic factors also influence water-quality conditions [46].

Each ecosystem that can be found in dammed water has their inherent specificity and variability. Microbes that colonize each of these ecosystems belong naturally to different genera, vary with their own ecological behaviour (autotrophic in photic zones of water, anaerobic in the sediments, psychrophilic in the deep thermocline). Native or indigenous microbiota of dammed waters includes, predominantly, psychrotrophic bacteria (*Acetobacter* spp., *Acinetobacter* spp., *Alcaligenes* spp., *Nitrosomonas* spp., *Desulfovibrio* spp. (sediments), *Geobacter* spp., *Rickettsia prowazekii*, *Coxiella burnetti*, *Wolinella succinogenes* and *Aeromonas* spp.), autotrophic bacteria (Cyanobateria), microalgae and protozoa [47].

Actinomycetes spp. and fungi can be abundant in surface water sources, including reservoirs. They can also grow in the water supply distribution systems, producing geosmin, 2-methyl isoborneol and other substances, resulting in objectionable tastes and odors in the drinking-water [48].

All those microbial agents may have irregular distributions and biomass depending on the abundance of organic matters; supply of nutrients for the heterotrophic microbes. The abundance of natural microbiota that colonize dams' waters is also dependent of other physical characteristics of the water: turbidity, temperature, pH, dissolved oxygen, salts. All this general microbial burden of the dammed freshwater may be partially assessed through analytic procedures, specially "total cultivable microorganisms", although, in fact, only the aerobic heterotrophic microbes will reveal its presence, using this laboratorial test.

The two main microbial processes that occur in freshwater lake habitats are the nitrogen and carbon cycle. Both these cycles affect the lives of the flora and fauna which co-share this habitat. The carbon cycle allows carbon to be recycled or reused throughout the biospheres and for all living organisms. It is essential for new life. Bacteria help breakdown dead and decaying organic matter. During decomposition, these bacteria will release carbon dioxide when oxygen is present.

Furthermore, the dammed waters may be accidentally contaminated by other exogenous microbiota introduced by human or animal activities (anthropogenic microbiota). Although these extraneous microorganisms are not dominant, they are the targets of the majority of the concerns. Its presence in dammed waters is always almost temporary, since they do not belong to the aquatic environment and, sooner or later, they will gradually decline. However, whenever they remain in water, they may pose a threat to the health of human (sanity), animal (zoo sanity) and vegetal (plant health).

Health issues associate with water and presence of exogenous microorganisms justify its systematic search as a way to manage the risks associated.

Microbial hazards of the aquatic chain may reach human and animal organisms through direct contact (skin, wounded skin, conjunctiva) (*Leptospira* spp., Poliovirus), respiratory route (*Legionella* spp.) and, more frequently, by the digestive via (table 1.3.).

Table 1. 3 - Most representative pathogenic microbes transmitted through drinking-water (adapted from reference [48])

Pathogen	Health significance[b]	Persistence in water supplies[c]	Resistance to chlorine[d]	Relative infectivity[a]	Important animal source
Bacteria					
Burkholderia pseudomallei	High	May multiply	Low	Low	No
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Yes
Escherichia coli – Pathogenic[f]	High	Moderate	Low	Low	Yes
E. coli – Enterohaemorrhagic	High	Moderate	Low	High	Yes
Francisella tularensis	High	Long	Moderate	High	Yes
Legionella spp.	High	May multiply	Low	Moderate	No
Leptospira	High	Long	Low	High	Yes
Mycobacteria (non-tuberculous)	Low	May multiply	High	Low	No
Salmonella Typhi	High	Moderate	Low	Low	No
Other salmonellae	High	May multiply	Low	Low	Yes
Shigella spp.	High	Short	Low	High	No
Vibrio cholerae	High	Short to long[g]	Low	Low	No
Viruses					
Adenoviruses	Moderate	Long	Moderate	High	No
Astroviruses	Moderate	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potentially
Noroviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Sapoviruses	High	Long	Moderate	High	Potentially
Protozoa					
Acanthamoeba spp.	High	May multiply	High	High	No
Cryptosporidium hominis/ parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naegleria fowleri	High	May multiply[h]	Low	Moderate	No
Helminths					
Dracunculus medinensis	High	Moderate	Moderate	High	No
Schistosoma spp.	High	Short	Moderate	High	Yes

A huge diversity of micro and macroorganisms are found living together in water, sharing metabolic activities to allow the life of each other. Some pathogens could only be discovered more recently thanks to the advancement of the methods of detection, like virus. Others only now are found to be possible pathogens in water, like some toxigenic fungi. In addition, there are, emerging waterborne pathogens, like *Campylobacter jejuni* [49].

Finally, there were some pathogens already describe, but only later associate as human pathogens. *Cryptosporidium* spp. was first described in 1907, recognized as an animal pathogen in 1955, but only as a human pathogen in 1980 [50].

The frequency of pathogens that are transmitted through the water routes is largely documented, and even in developed countries they may be sporadically found, despite of the systematic water treatments. E.g. in 1993 in Milwaukee, USA, 400 000 people suffered gastroenteritis after having drunk water contaminated with *Cryptosporidium* cysts. In 2000, people felt ill in Walkerton, Canada, because of a contamination of drinking water with *E. coli* O157:H7 [34].

The probability of pathogenic microorganisms spread through water is increasing because of the effect of agricultural magnification due to irrigation of fields. The fresh food demand is increasing, due to population growth, facility of people travelling and climatic changing [51]. Examples of enteric waterborne emerging pathogens include caliciviruses, *Helycobacter* spp., *Mycobacterium* avium complex (MAC) and the protozoa *Cryptosporidium* spp., *Cyclospora* spp. and *Toxoplasma* spp. Systematic surveillance, monitoring and development of methodologies are needed to detect such threats in useful time [34].

Waterborne diseases are a major problem in developing countries because people are more exposed to waterborne pathogens due to accessing difficulties of treated water (potable). They are more susceptive to recurrent infections, frustrating the economic development [52].

Microbial contaminations of water can be originated from a wide range of sources, including urban and industrial effluents or agricultural runoff. Humans and animals are the primary sources of agents that may compromise “water quality”, contributing to its degradation.

Microbial contamination relates to the introduction of exogenous saprophytic or harmful bacteria, viruses or protozoa, collectively known as pathogens, into a water source.

Inorganic and chemicals contamination may also occur, such as: heavy metals, polycyclic aromatic hydrocarbons (PAH), dioxins congeners (PCDD, PCDFs, PCBs), pesticides, and many “undesirable substances” (bitoxins) produced by aquatic microorganisms, seeping into drinking water sources from geologic strata, soil or rain [53].

The exact sources of infectious diseases were uncertain before the discovery of microorganisms, but after its recognition, the importance of its nosology have surpass all the initials expectations [54]. Many of them may remain infective in water for long periods, traveling from the initial source. In urban watersheds, fecal indicator bacteria are significantly correlated with the local population density, which can be related with the source [55].

Nowadays, there is a more accurate public perception concerning health risks. Populations assume that, whenever a pathogen is found in drinking water a corrective action need to be undertaken.

1.4.1. Bacteria

The knowledge of native and exogenous bacteria that stands in the waters is imperative, because they are the key to life sustainability. Precautionary measures or urgent action plans, are not possible to put in place without that assessment.

Bacteria are unicellular prokaryotic organisms with a variety of body morphologies; most common are bacillus (cylindrical, rod shaped), coccus (spherical) or spirillum (helical rods) and some are pleomorphic. Some bacteria are photosynthetic, others oxidize inorganic compounds, and still other bacteria generate energy by breaking down carbohydrates in a respiratory process. A number of bacteria require oxygen (aerobes), others cannot tolerate it (strict anaerobes). Bacteria can also grow either with or without oxygen (facultative anaerobes).

Along the evolutionary process, bacteria had adapted to aquatic environments as permanent habitat, since nutrients were available. Bacteria is a very frequent organisms present in waters. Due to their diversity, plasticity and adaptive metabolism they may colonize different environments in extreme conditions (volcanic lakes, or glacial seas). They are referred as had being the most primordial organisms in the beginning of life on Earth (with Archaea), proved through fossils vestiges [56].

Aquatic bacteria are known since the earliest microbiological searches, being predictable that all species of the aquatic ecosystems have not been yet described [57]. A very small number of bacterial species, native of the natural aquatic ecosystems, have been incriminated in animal and human infections (*Leptospira* spp., *Legionella* spp., *Aeromonas* spp. *Plesiomonas* spp., *Acynectobacter* spp. and *Vibrio* spp.) (Table 1.3).

When water intended for drinking presents a very elevated level of general microbiota, the probability of containing microbial agents with potential pathogenicity for human is higher. However, not only humans are exposed to hazardous contaminations of waters, all the natural species, living in those waters, may be at risk.

Multiple descriptions on the third quarter of the nineteenth Century, attesting the role of the drinking water as a vehicle of *Vibrio cholerae,* were crucial to set out all the strategic measures need to ensure safety of water used for direct consumption.

The early discovers represent a crucial step for establishing Microbiology as a true science and they have amply demonstrated its benefits to mankind. It has been possible to find a new approach in the last 1.5 centuries, step by step, developing new analytical procedures. They are more accurate, sensible, refined laboratorial methodology and adequate strategies to manage the microbial risks associated with water.

It is an issue of major concern, for all parts, that drinking water can be contaminated with pathogenic bacteria: consumers, producers, regulators and managers of risk.

The most important bacteria able to cause gastrointestinal diseases transmitted through water are: *Vibrio cholerae*, *Salmonella* spp. and *Shigella* spp. These diseases are mainly transmitted through water contaminated with feces of patients or healthy carriers. However, the presence of pathogenic bacteria in water is, in general, sporadic, fortuity, occasional or erratic. Levels of contaminations are low and, sometimes, the culture of those bacteria for isolation and characterization is not a straightforward task [58].

Some of the waterborne contagious diseases do not express clinical signs. That may be the explanation for some facts related with mild symptoms in humans. Signals are not always or easily attributable to water contaminations, being even ignored by human, due to its episodic course, disappearing without requiring specific treatments. Some waterborne infections are mild, not requiring complex medical assistance. Some example may be referred:

- *Campylobacter* spp. infections, due to bacteria that frequently cause mild gastroenteritis, generally auto-limiting, easily avoided by sanitation [59].
- *Plesiomonas shigelloides* infection is also generally suspect and only revealed when outbreaks are investigated; the most common event is the lack of information concerning its frequency and number of affected persons. The increasing number of cases, reported in recent years, is a clear evidence that it has been overlooked by many epidemiological studies [60].

Pathogenic virus, protozoa or bacteria having drinking water as vehicle are, most of the time, not directly surveyed by the authorities, uncharged of applying the regular control system of water sanitation. These pathogenic agents are accessed indirectly, through the use of laboratorial procedure that is focused on indicators. Direct search of aquatic pathogens is, generally, reserved for epidemiological surveys when outbreaks occur and their investigation is essential to establish causality links.

1.4.1.1. Bacterial indicators

It is important to refer the laboratorial use of some bacteria as indicators, agents that are capable of predict the presence of exogenous pathogens. Particularly those that are introduced in the water through fecal contaminations. The knowledge of its characteristics is relevant. Its detection in water samples is a step forward in the early signalization of the possible presence of a pathogen.

Monitoring each pathogen would be not practicable for economic and time-consuming methodologies. These detections are not consistent with a routine procedure. Attending to these reasons, routine water microbiological analysis does not include the direct detection of pathogenic agents. Nevertheless, water must be free from pathogenic microbes so that may be considered safe. The conciliation of the two principles was met by testing for indicator bacteria [58].

For these reasons, a brief resume about the meaning of an indicator and those that are more frequently used in water not only seems obvious but are also critical to reflect about their importance and impact in the water tests done around the Globe. With incomplete data, it is more difficult to avoid human infections.

The use of indicator organisms was suggested in 1880, observing that *Klebsiella* spp., a common agent of the respiratory and urinary tracts, was also present in human feces and in water. Methodologies allowing to distinguish *E. coli* from other intestinal bacteria emerged in 1900s. Analytical procedures concerning those methods are still applicable nowadays [61].

Indicator microorganisms can be assessed using several methodologies, adapted to different economic capacities, because they are easy to detect and quantify. Furthermore, the use of indicators has economic benefits since its protocols for detection and quantification are lower-cost methods than those necessary to detect the pathogens. This is another advantage that makes the procedure useful for poor regions of the Globe.

The characteristics of an adequate indicator for fecal contaminations may be resumed as follow [62]:

- Not multiply in the environment (aquatic ecosystems)
- Be absent in unpolluted water and present only when fecal contaminations occur;
- Be present in higher levels than the pathogenic microorganisms that it indicates;
- Respond to natural environmental conditions and water treatment processes similarly to the concerning pathogens;
- Not reproducing outside of the host;
- Be easy to isolate, identify and enumerate;
- Not be too expensive and difficult to search;
- Not be a pathogenic microorganism.

It is difficult to find indicators in use able to meet all these criteria simultaneously [63]**.**

To use an indicator is a way of indirect measuring of pathogens without assessing and quantifying them. It can be inferred that the pathogens cohabitant of its ecosystem may be, or has been, present in the water, when an indicator is found above a critical level. The presence of an indicator does not corresponds, for sure, that pathogens are present, but it allows signalizing fecal materials in the water.

The number of pathogen agents associated with the concentration of its indicator is a function of the disease incidence at the time of the exposure to water contaminated with the fecal material. But a specific water in which, a chosen indicator, is not present or detect is not necessarily free of pathogens; technically there is no indicator that fully represents the presence of a pathogen [64].

There are four principal bacterial indicators that have commonly been used for a long time as fecal indicators: total coliforms (TC), thermotolerant coliforms*, Escherichia coli* and *Enterococcus* spp.. Each one of them has their own advantages and disadvantages. They are depended on the environment in which the sample is obtained. Certain indicators are more appropriate for specific sampling locals.

1.4.1.2. Total coliforms (TC)

The coliform group is an adequate indicator for the general hygiene level. When they are present in a higher number and concentrations than other pathogenic bacteria, allows estimating the probability of finding pathogens. The “total coliform” standard is still in use, e.g. in drinking water, since it is felt to be a very conservative risk management tool [65].

Historically, the term “coliform” mean “all of the lactose-fermenting species of the *Enterobacteriaceae* family (taxonomically meaningless)”, commonly found in the feces, excluding genera of non or slowly-lactose-fermenting bacteria, some enteric pathogens and bacteria living naturally in the environment (phitophylic) [66].

Lactose fermentation is a characteristic of considerable diagnostic importance to distingue among the different groups of Gram- enteric bacteria. The enzyme ß-galactosidase can react and the galactoside permease facilitates lactose entry into the cell [66].

Two major practicable definitions of coliforms have been distinguished: “total coliforms” and “thermotolerant coliforms”. Coliforms are gram-negative rod-shaped bacteria, non-spore forming, oxidase negative, anaerobes facultative that can ferment lactose (enzyme ß-galactosidase), in a matrix

with 1.5% of bile salts, producing acid and gas in 48 hours at 36 ± 2 ºC. Some are also found in soil (*Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp.), others in vegetables (*Klebsiella* spp., *Enterobacter* spp., *Ewinia* spp., *Serratia* spp., *Hafnia* spp., *Providencia* spp.). Coliform bacteria may be opportunistic pathogen for humans and animals when present either in the gut (enteropathogenic, enterotoxic, enterohaemorragic and enteroinvasive *E. coli*) or in other parts of the body [66].

The presence of "total coliforms" in a specific drinking water can indicate that the treatment was not efficient (are sensible to chlorine, iodine, ozone, UV), pointing to a deficient quality of the water.

It is possible that this procedure detect bacteria that are not members of the family *Enterobacteriaceae*, like some species of the genus *Aeromonas* spp., also lactose-positive. For these reasons, another group, "thermoteolerant coliforms" has been established in an attempt to separate the "total coliforms" into those of fecal and non-fecal origins [66].

1.4.1.3. Thermotolerant coliform

In 1948, Mackenzie et al. distinguished 'fecal coliform' from 'total coliform' through thermotolerant incubation (44.5 ± 0.5 ºC) (lactose$^+$, bile salts$^+$) and indole-positive reaction [67].

The thermotolerant coliforms are a subset of the previous describe group, having theoretical characteristics more useful as fecal indicators. They are able to grow at higher temperatures and are expected to come from the intestinal tract of warm-blooded animals, including humans [68]. They are able to ferment lactose producing acid and gas within 48 hours at 44.5 ºC but, using this methodology, several coliforms were shown not to be always represented a fecal contamination (*Klebsiella* spp.). So, the term "thermotolerant coliform" is preferable to the previous name (fecal coliforms) [67].

They are defined nowadays as gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose, producing gas at 44.5 ± 0.5 ºC within 24 ± 2 hours, in the presence of 1.5% of bile salts. Alternatively, possess the enzyme j1-D-galactosidase, which is capable of using a chromogenic galactopyranoside substrate for growth [69].

They come from the intestinal tract and, when released in water, they lose their viability after a few weeks (it is difficult to discriminate between human and animal source). They represent a fresh fecal contamination, but several factors may affect the results (for example, *Klebsiella* spp. associated to excressions of the respiratory or urinary tracts). Microbial analyses of water based only in this indicator should be avoided. Even with this limitation, one significant attribute that is pointed is the fact that as a regulated tool had proved to be efficient [64].

They resist in the water similarly to bacterial pathogens of the enteric environment, but are restricted indicators of protozoan or viral contamination [65]. In tropical waters, they proliferate and may be detectable at high levels, which do not reflect the original extent of the fecal contamination [70].

"Thermotolerant coliform" tests are applied to surface and ground water contamination, sewage treatment systems and general monitoring for safety of natural waters, but is not considered a substitute for the total coliform test in the examination of potable waters [66]. To reduce the possibility of false-positive results, a confirmatory test for *E. coli* is recommended.

Coliform bacteria of any kind should not be tolerated in 100 ml of finished (treated) drinking water.

1.4.1.4. *Escherichia coli*

Eijkman suggested *E. coli* to be imperative for bacterial assays in 1904, due to its thermotolerance and fermentation ability. US EPA recommends *E. coli* since 1986 as the most adequated indicator for assays of fresh water, and it is considered the best indicator for the presence of potentially pathogens of enteric origin [55].

E. coli is the only agent on the coliform group that is directly associated with fecal contamination and not frequently found widely in the environment. A set of studies has shown clearly that *E. coli* is an inhabitant of the gastrointestinal tract, while *Klebsiella* spp., *Citrobacter* spp. and *Enterobacter* spp. were found only in small numbers (when present) [64].

One pathovariety of *E. coli* has received much attention, serotype 0157:H7, because of its pathogenic potential, but, as a paradox, this particular biovar does not growth at temperatures superior to 41 ºC. Not all of the strains of *E. coli* are pathogenic. Tests for detecting its presence in water found mostly non-pathogenic *E. coli* strains. This condition allows to state that the search of *E. coli*, in the water, has few risks associated.

Whenever *E. coli* is detected in water, it means a recent event of fecal contamination and the possible presence of any enteric pathogens. This indicator is believed to allow a correlation with *Salmonella* spp.[71], one of the most common cause of waterborne outbreaks (typhoid fever is now rare, in what proves the success of this indicator). Although, in the literature, some contradictory results has been referred, with some studies showing no correlation [71, 72]. In tropical regions, the relation can be perturbed, *E. coli* may be present and multiply naturally in waters, like other coliforms, assuming an ecological behavior that is not applicable to temperate climates [74].

For bacterial fecal pathogens (*Salmonella* spp., *Shigella* spp., pathogenic *E. coli* and *Campylobacter* spp.), many studies have shown how *E. coli* helps to predict them, in both surface and groundwater's [75]. Several studies proved the correlation between the presence of *E. coli* and viruses or parasites in waters. Some cases report this relationship, specifically with *Giardia* spp. [76]. The presence between *E. coli* and enteric viruses in surface water sources was also found, known to be contaminate by human feces [77].

The World Health Organization states, *'Water must be examined regularly and frequently because pollution is often intermittent and may not be detected if examination is limited to one or only a small number of samples. For this reason, it is better to examine drinking water frequently by means of a simple test rather than less often by several tests or a more complicated test.*' Furthermore, the WHO states, *'Examination for fecal indicator bacteria in drinking water provides a very sensitive method of quality assessment*.' *E. coli* best fulfills these conditions [78].

If is used a single parameter as fecal indicator, instead of analyzing drinking water for other possible pathogens, *E. coli* testing can be a very simple and not too much expensive possibility.

1.4.1.5. *Enterococcus* spp.

Winslow and Hunnewell, in 1902, suggested fecal streptococci to be used as indicators for fecal contamination of waters. WHO report, in 1997, on various species of *Enterococcus* spp. and *Streptococcus* spp. to be included as fecal streptococci [67]. The term *Enterococcus* spp. had been used to describe fecal streptococcus and organisms that have similar laboratorial behavior of *Streptococcus fecalis*.

Enterococcus spp. are found worldwide in feces from adults and infants being the species *E. fecalis* and *E. faecium* the predominant ones. These microorganisms are present in all mammals´ colon, but less numerous than *E. coli*. In the human body is inhabitant of the gastrointestinal tract (can be isolated from other parts of the body in a small number) in high concentrations [78].

Differences in concentrations of “fecal coliforms” and “fecal streptococci” in the same water sample (the ratio FC: FS) were reviewed and ported as relevant for the differentiation of the contamination source. In human feces, the FC: FS ratio is reported to be >4, in contrast to a ratio of <0.7 in animal feces. It has fallen due to the differences of survival ratio of these two group of microorganisms, as such this ratio should not be relied on [74].

The genus *Enterococcus* is nowadays describe (along with the already described characteristics) as gram-positive “coccus” species, facultative anaerobes (chemo-organotrophs), associated in chains, in 1.5% Bile salts. They are considered strict fermenters, lacking a Krebs cycle respiratory chain (catalase - negative). This last characteristic is helpful in the identification of *Enterococcus* spp. found during the monitoring studies, allowing differential test.

Enterococcus spp. survive and grow in different environment due to their ability to resist in more severe conditions than coliforms. They are recovered from water, soils, food and numerous animals, including insects. An important characteristic of the genus *Enterococcus* is that they are relatively salt-resistant (< 6.5% NaCl), which makes it an adequate fecal indicator of estuarine and ocean waters. Several studies confirm that fecal streptococci are more persistent in aquatic environments than fecal coliforms [74].

1.4.1.6. Total cultivable microorganisms

The heterotrophic bacteria are a much extended group of microbes that are able to use directly organic carbon sources to grow. They colonize all ecosystems on which life is possible, including the aquatic. The number of bacterial species and bacterial population present in water are proportional to the amount of organic matter available. Its presence in water is usually revealed through the procedure applicable to the determination of “total cultivable microorganisms”. This analytical procedure, in fact, does not reveal “all” bacteria present in water samples, since it only allows the appearance of colonies generated by the growth of aerobic mesophilic bacteria and some fungi [64].

The procedure excludes: extremophile microorganisms, anaerobic bacteria, bacteria requiring special nutrient factors and viable non cultivable microorganisms. It is assumed, however, as “standard plate count”, “aerobic plate count” or “total plate count”. The analytical procedure does not allow to distinguish between microorganisms that are indigenous of the aquatic ecosystem and those introduced by exogenous contaminations.

Since the late 19th century, this parameter has served to address the purity of a water source and it is not thought as a possible contamination by pathogens. It not indicates a sanitary risk, but only an information concerning the general level of organic matter present in the water. This information is useful to alert for eventual deterioration of the water quality, as well as to confirm the efficiency of water treatments (slow and sand filtration, chemical end physical treatments). If it contains less than 100 bacteria / 1 ml, the water may be suitable for drinking [79].

Total microbial burden of drinking water is measured indirectly, using “colony forming units”, in a solid medium (agarose). Whenever the number of those colonies is superior to the maximum limit, it may reflect a possible problem with the adequacy of treatments applied to water.

Fecal contaminations had been correlated with the total number of colonies forming units by millimeter of water, either with which develop at 37 ºC and, principally, with the saprophytes that grow at 22 ºC [69]. Bacteria have a relatively higher resistance to biocides (chemical disinfectants) than with other treatments (physicals). The use of total cultivable microorganisms, as a single parameter to assess water safety, may conduce to inadequate judgments [80].

Cultivable microorganisms, like “thermotolerant coliforms”, is an adequate parameter to be used in the routine monitoring of drinking water quality. *E. coli* remains the parameter of choice for the majority of the fecal contaminations tests, with thermotolerants as an alternative. *Enterococcus* spp. are alternative parameters for fecal contamination or for monitoring the distribution/storage systems.

1.4.1.7. Limitations

The systematic application of those parameters and the use of its criteria as a tool, proved to be effective in the control of the drinking water quality for many decades.

These parameters are recognized as guidelines for drinking water by the WHO. Even with all the theoretical limitations, because they are easy to perform. Nevertheless, it is important to reflect about the limitations of these procedures, having always in mind that microbiological analysis of the water is still incomplete and with chances to be improved.

First, the most important fact concerns to the question that none of the indicators used for routine monitoring of drinking water cannot express information to all eventual pathogens present in the water. Namely those of the aquatic environment: *Leptospira* spp., *Legionella* spp., *Vibrio* spp., *Aeromonas* spp.. New and more accurate indicators are always under scrutiny and search development.

Some of the previously referred indicators are typical inhabitants of the human and animal intestine and are assumed as being bacteria that only grow in environments rich in nutrients. For example: *E. coli* is uncommonly found in water, but studies along 15 years demonstrate the survival of *E. coli* in long-term starvation conditions. This affects the interpretation of the results in the routine tests. Maybe *E. coli* survives for longer periods after the contamination [4].

The use of an indicator in recent years, like the “total coliforms”, has been questioned and considered an unreliable indicator of fecal contamination, because they are capable of growing in the environment and drinking water. It was found that 61% of the total numbers examined over 1000 strains of coliforms were non-fecal in origin. This confirms that the presence of coliform bacteria may be a natural factor in nature and does not indicate a health risk [63].

When samples are collected in a point of the water column faraway from human influence, and *E. coli* is found, the source of contamination may just be birds, wildlife, or livestock.

E. coli seem to be more resilient in turbid waters, because UV radiation (sunlight) and its bactericidal effect do not take place. Water temperature and pH also influenced its levels. In warmer environments, total microbial counts are higher (in general), due to its longer survival at those temperatures [55].

All these factors enhance variability, making difficult to forecast the results. Concerning *Enterococcus* spp. tests, it appeared to be sufficient in the past to presumptively identify this group of agents. It has come lately to light that other less commonly encountered gram-positive cocci may also give positive reactions in some of these tests. For example, some cultures of *Lactococcus* spp., *Aerococcus* spp., *Pediococcus* spp., and *Leuconostoc* spp. are bile-esculin positive or can grow in 6.5% NaCl or both [81].

A study, published in 2005, put in cause the validity of using indicator organisms (total and fecal coliforms, enterococci, *Clostridium perfringens*, and F-specific coliphages) to predict the presence of pathogens (infectious enteric viruses, *Cryptosporidium* spp., and *Giardia* spp.). No strong correlation was found for any binomial combination indicator-pathogen [82].

In relation to “total coliforms”, for example, some pathogens persist in water for longer than coliform bacteria, including enteric viruses, which were detect without any signs of other conventional fecal contamination [83].

One of the problems more frequently referred is the lapse of time required to culture microorganisms and obtain a result. The “Standard Method for The Examination of Water and Wastewater”, a reference text, refers that the most rapid conventional methods take, at least, 24 hours until a result is obtained. This implies that when the result is obtained, probably the water has been already consumed without adequate access to information concerning its safety.

To prove a correlation between an indicator and a specific pathogen or a source of contamination, extensive and detailed analyses are required. Inconsistencies between the results pointed by different works are often found.

Work conditions, the sampling collection, times of samples arrivals to the laboratory and the environment can significantly influence the final results. It is fundamental to follow standard and validated procedures and respect the methods to avoid a minimum variance. Using accredited methods, enforced in all official laboratories it can reduce the variability of the results and may allow the exchange of comparable information.

1.4.2. Cyanobacteria

Phytoplankton are free-floating microorganisms found in salt and freshwater. It is compose principally by the major taxonomic groups: green algae, diatoms, dinoflagellates, and cyanobacteria [84].

Algae is a term sometimes used to describe both green algae and cyanobacteria. Green algae belong to eukaryotes, divided into groups by the color they reflect when expose to sun light. Cyanobacteria are a prokaryotic microorganism (frequently know as blue - green algae) that co-exist

with green algae in aquatic environments, forming communities within the water column (pelagic) or attached to bottom surfaces (benthic).

Cyanobacteria are closer to bacteria in terms of cell structure. They are gram-negative bacteria (eubacteria or true bacteria), which class include 150 genera and about 2.000 species. They can grow in numerous ways, as single cells, single cells in colonies (may be packed in a mucilaginous sheath, e.g. *Microcystis* spp.), or single cells in filaments (floating mats or free-floating strands). Some filamentous genera contain nitrogen-fixing heterocysts cells, e.g. *Anabaena* spp., that givens then a better chance of survival when nitrogen levels limit the growth of other algae or microorganisms [85].

Other advantage of many cyanobacterial species is their control over the position in a water column throughout gas vacuoles, a distinct ecologic advantage over other planktonic species [86].

It is a very ancient group of microorganisms, as one of the oldest fossils on earth, believed to be more than 3.5 billion years. Cyanobacteria habitats are quite variable, ranging from hot springs to temporarily frozen ponds in Antarctica. They occur worldwide, frequently in calm and rich nutrients water, adapted to almost every environment in the planet. Even with the changing environmental conditions, the investigators appointed that they will continue to survive [87]. E.g. climate rising temperature (a global question nowadays) favors cyanobacteria, because their optimal growth usually occurs at moderate temperatures, like 25 °C [88].

Other favorable factors for the adaptation to freshwater systems seen in cyanobacteria (contrarious to others phytoplankton groups) are vertical thermal stratification and alterations in seasonal and interannual weather patterns [84].

Cyanobacteria are autotrophic, primary producers making up the bottom of the food web chain [89]. Many organisms rely on them (directly or indirectly) as a food source (zooplankton feeds from them) [90]. E.g. they serve as food for mosquito larvae, vectors of tropical diseases. Cyanobacteria are extensively spread in mosquito habitats. Their abundance and distribution influences the biological control of mosquito larvae. They can be a relevant factor in controlling vectorial diseases [91].

The purple and the green sulfur bacteria, in contrast to their closest relatives, produce oxygen (only bacteria that are known to exert this function) due to the photosynthetic activities. They are appoint as the responsible for the production of oxygen in the beginning of life in Earth. Cyanobacteria have chlorophyll (use the sun light as an energy source) varying in color from green through blue-green to red. Chlorophyll-a is a pigment generally use in quantification of all the photosynthetic organisms present in water-bodies [85].

They provide food and oxygen to nearly all live, thanks to their photosynthetic activity. Not only has this important function, cyanobacteria also regulated inorganic carbon (carbon dioxide) in the atmosphere. Carbon dioxide and water molecules are use in the carbon cycle (carbon fixation, part of the biological carbon pump) from photosynthesis to make sugar and energy. This process helps to normalize the global temperature. If they decrease significantly, they may alter the CO_2 level in the atmosphere and could have an effect on the world climate [92]. .

1.4.2.1. Blooms and toxin production

The organic enrichment of dams´ water and lakes is call eutrophication. It is possible to result in blooms of cyanobacteria, if there is an increase in the water temperature. The presence of inorganic salts like phosphates, ammonia and nitrates are also relevant. Blooms are mass development of cyanobacteria (high-density populations) floating on or near the water surface reaching cell numbers > 10^6/ L [93].

The worldwide frequency of algal blooms, both marine (called red tides) and freshwater (called waterblooms or CyanoHABs), is steadily increasing (there is an international consensus [94]), with the severity and duration also rising. The microorganisms implicate are not exactly know and the variety of species found in different places are not constant.

Historically, the oldest cyanobacterial bloom register was in 1878 in Lake Alexandrina (South Australia) caused by cyanobacteria *Nodularia spumigena*, where some sheep has died after the intake of water from a reservoir [95]. It was finally observed a direct correlation and was constituted a department dedicated to study their influence. Reports had started to appear from many countries around the world.

In fresh water environment, the occurrence of toxic cyanobacterial blooms presents problems for treatment, management and regulation of the quality of drinking water supplies, posing a threat to humans and animals [94]. Cyanobacterial blooms comprised mainly of the genera *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrixx*), *Nodularia* and *Aphanizomenon* [96].

The causes to this expansion are still unrevealed, but alteration of the water quality seems to be an important factor [97]. E.g. reservoirs in Germany, near human populations, are enhanced with nutrient loading, mostly from agricultural runoff and domestic wastewater, leading to cyanobacterial blooms (frequently from the species *Planktothrix rubescens)*. This is so a usual phenomenon, well-studied there, that was accepted as the most important contaminant in the Weida Reservoir (provides potable water to the population of East Thuringia) [98].

A natural freshwater lake is very rare in South Africa. The demands for drinking water are promoting the development of artificial lakes and dams. In a study about the Vaalkop dam, which is used to produce potable water, the frequency of cyanobacteria's (seasonally developed) and cyanotoxin production were investigated. It revealed that the risk of cyanobacteria bloom formation are higher when favourable environment conditions were found (nutrient loading and temperature increase), being microcystin the most common cyanobacterial toxin [99].

If these blooms did not had dangerous consequences, they could be unnoticed, but the effects on freshwater fauna were demonstrated "in vitro". All the important fresh water herbivorous phyla suffer negatively by cyanobacterial blooms, and protozoa/terrestrial animals (e.g. mammals) accidentally consuming the water are affect too [100].

The harmful effects of the blooms have direct or indirect economic consequences resulting in the reduction of biological diversity, oxygen depletion and largely perturbing the quality of the drinking water (an unpleasant odor and taste), blocking the filters (increasing the maintenance costs) [97].

The most crucial on impact water quality from cyanobacteria are the production of secondary metabolites (low molecular weight organic molecules), very dangerous toxins named cianotoxins.

The contact of humans with these toxins are through drinking or bathing in contaminated water. The symptoms range from skin irritation, stomach cramps, vomiting, nausea, diarrhoea, fever, sore throat, headache, muscle pain, and liver damage. They are referring by different names depending on the affected organ in humans (hepatotoxins and neurotoxins), but all share as characteristics an absence of odor and taste [101].

The importance of these toxins is still in debate with various theories erasing: 1) a sub-product of metabolism (because is a combination of different proteins); 2) a factor to compete with other microorganisms; 3) a functional protein to prevent the damage of its own structure; 4) a target protein that only is release after the cell is dead to alert and attract other cells (that helps the survival cell to resist the stress) [102].

Many natural occurring cyanobacteria species are well known to produce toxins but not all strains form toxins. Hepatotoxins (liver effects) are produce by some strains of the cyanobacteria genera *Microcystis*, *Anabaena*, Oscillatoria, *Nodularia*, *Nostoc*, and *Cylindrospermopsis*. Neurotoxins (nervous system effects) are produce by some strains of *Aphanizomenon* spp. and *Oscilatoria* spp. [103]. Recent works correlate the occurrence of cyanotoxins with the frequency of neurodegenerative diseases (Amyotrophic Lateral Sclerosis, Parkinson's disease and Alzheimer´s disease) [104].

The most widespread of the cyanotoxins are the peptide toxins in the class call Microcystins. There are about 80 different microcystins (at least), including Microcystin-LR, which is consider one of the most toxic. Microcystin is a hepatotoxin, commonly found in the genus *Microcystis* and it was found to be produced by other genera, including *Anabaena*, *Nostoc*, *Nodularia* [103].

There were deaths of dogs and birds by the consumption of surface water with cyanobacteria blooms. One of the most important cases reported were human deaths (this is infrequent) of patients exposed intravenously to water containing microcystins in a kidney dialysis center in Brazil in 1996 (50 deaths). This water had concentrated toxins, coming from a dam [105].

What promotes the toxin production is, as with alga blooms, not truly understand. Factors influencing toxin production have not been conclusively elucidate, and connections between alga blooms, cell numbers and toxin levels are usually not significantly related [86].

Recreational and drinking waters were investigated in Seoul, South Korea, to find a correlation between cyanobacteria biomass, chlorophyll a and total microcystin. The total microcystin value was below the WHO guideline danger level, while chlorophyll a and cyanobacterial cell counts were within the 'cautious' and 'alert' level for drinking and recreational water [106]. This shows how analyses can induce an investigator to be alert, when the level of cyanobacteria microcystin are not dangerous, leading to an overprotection and unnecessary costs. The effect of long time exposure to low doses is not elucidated.

In a study to characterize Polish freshwater bodies (21 samples from 5 different provinces), the occurrence of several toxin producing species both and non-toxic strains was frequent. A correlation between cyanobacterial biomass and toxin concentration was not obtain. It was proven that environmental conditions have only minor or indirect effects on toxin production and concluded that only factors that induce the grow of toxic strains can affect the water quality [107].

Cyanobacteria that lack toxins can be used as food supplement or in alternative medicines. They have therapeutic value, medicinal active components can be obtained to treat malnutrition, cancer

[108] and viral infection. E.g. several cyanobacteria species were isolated and purified in an Egyptian rice field (2014), that could be use in designing an effective Hepatitis C virus medication. The absence of genes encoding to production of toxins were analysed by PCR techniques [109].

An important application is the use of cyanobacteria photosynthesis to produce carbohydrates, fatty acids, or alcohols as renewable sources of biofuels. Modern (engineered) cyanobacteria: 1) grow fast; 2) do not compete for agricultural lands and resources; 3) efficiently convert excessive amounts of CO_2 into biomass; 4) many species are easier to manipulate genetically than eukaryotic algae and other photosynthetic organisms [110].

Cyanobacteria blooms may arise at a rapid velocity without time to avoid invasive growth if a certain point is achieve. This demand a threshold values in the detections by the competent agencies that obliges to preventive measures the sooner possible. The procedure for identification of cyanobacteria is by laboratorial isolation in cultures medium, direct visualizations with concentration in an Utermohl chamber, with possible calculations of biovolumes. These are still the most reliable methods.

Molecular methods (with gene sequencing) will improve the identification of cyanobacteria species, reducing the time needed for laboratory growth of the cyanobacteria cultures [111].

There are several methods to detect and quantify cyanotoxins. Chemical methods based on High-Performance Liquid Chromatography (HPLC), immunological assays such as the Enzyme-Linked Immunosorbent Assay (ELISA) are used to detect and also quantify its concentration [104].

A provisional value was calculated by the WHO in drinking water. A guide value that has been apply in several countries; it states a maximum value for microcystin-LR of 1 µg/ L. Each country use these values (and other parameters), ranging between 1 and 1.5 µg/ L. Some use this as default value for other microcystins as well [112].

Eutrophication does not have a solution because each region has different chemical and physical characteristics [113]. Dangers to human health arise when blooms occur, because of the possible contact with toxins. The best way to prevent a bloom formation is to avoid the contamination by toxic cyanobacteria, control sources of nutrients to reaching the water, or avoid the water column stability (that allows cyanobacteria to developed) through artificial mixing (very expensive) [114].

Active carbon and ozone are capable of partially retain or eliminate cyanobacterial toxins but only in places with previous blooms routines observed (not as a normal procedure) [114].
Biological processes or addition of chemicals are low technology demanding, with little or no maintenance running costs, but produce potentially harmful products to eliminate cyanobacteria [87]. Phosphorus is the major nutrient entering the dams from the rivers. Preventing this nutrient (or others) from entering is the best way to achieve a long term solutions [113].

1.4.3. Virus

Virus are small microorganisms, submicroscopic, ranging from 20 to 300 nm on size. One very important characteristic to understand the cycle of life of this microorganism and its survival, is the fact that it depends entirely of a host to sustain its own activities. They are obligate intracellular parasites, consisting of a nucleic acid genome that may be double- or single-stranded DNA (DNA virus), or double- or single-stranded RNA (RNA virus), another specific characteristic. The genome is protected by a

protein coat, and some have an outer layer called envelope (lipoprotein). A virus particle is the viral genome surrounded by a capsid (other coating protein).

Viruses depends on a host, infecting all the other microbiotic (e.g. fungi and bacteria) or macrobiotic (e.g. plants and animals), to multiply until the moment some are expelled from the interior habitat to the environment. Ones will survive till attaining a route to reach another susceptible host, but not all will be successful [115].

Virus particles were estimated to achieve billions per liter of water, surpassing the values calculated to bacteria or others microorganisms. Sometimes virus are not consider organisms depending on the author and theories followed, which will not be discussed, because they do not metabolise or reproduce outside a host cell.

Virus present in water are so specific to a certain organisms that it can be said that only human viruses are possible agents of waterborne disease [116]. The disease is caused by disruption the normal cell function, repressing essential proteins (inhibit synthesis of the normal components), weakening the cell membranes, plus inducing autolyse. Some proteins from virus can be toxic to human cells, with the body's immune defenses killing the host-infected cells [117].

To classify virus several parameters are use: the shape, size, type of genome, among others. E.g. DNA virus are the herpes viruses (chicken pox, cold sores, and painful genital lesions) and the poxvirus (smallpox). RNA viruses include rhinoviruses (common colds), rotaviruses (gastroenteritis), and retroviruses (AIDS and several types of cancer) [117].

Virus are a biggest laboratorial challenge when comparing to bacteria, due to their small dimension that allows them to escape water filtrations. They become contaminants of water, if it is not ensuring their complete removal, with the possibility to promote health damages [118]. The utilization of chlorine in water treatment can inactivate the majority of enteric viruses, with low costs associated and the additional advantage of reducing the level of other microorganisms also susceptible. Systems based in Ultra violet (UV) radiation proved to be useful, requiring dose changing with the virus in question; for a 4-log reduction: adenovirus is 226 mJ/ cm^2, 56 mJ/ cm^2 for rotavirus and 39 mJ/ cm^2 for Hepatitis A virus (HAV) [119].

The actual burden of waterborne viral infections is still hard to figure owing to technical limitations in pathogen detection, scarce data on environmental epidemiology, difficulties in determining the source of infection, occurrence of unapparent infections and because some diseases can be transmitted from other sources [118]. The enteric viral outbreaks could be higher than the reported value, due to these problems, and responsible for the outbreaks under the “undetermined” etiology [120].

Virus are considered a principal cause of water-related disease via recreational or drinking water, via irrigation and contamination of shellfish growing areas, even underestimating the value - their resistance can sometimes further confuse their association with the source or disease [121]. Their multiplication is inhibited when they are living freely in water, but the capacity to survive is maintain and some virus have been found to sustain from 2 days to 6 months or more [122]. Other studies reveal that they remain infective for 120 days in freshwater (130 days in seawater) [120].

The longer periods they survive in the environment improves the probability of entering a human being, contaminating previous safe and pure waters, and traveling long distances from the source [123].

They are difficult to be detect by water analyses, when in low concentrations (diluted in a reservoir, like a dam), but with a number that still can promote diseases [83].

The main enteric viruses' source is contaminated water from sewage, which carries over 100 virus species, but only few were demonstrated that water is the main source from them. The viral outbreaks (e.g. gastroenteritis) have been correlate with drinking water, irrigation, aquaculture, food processing, or recreational purposes [124]. It was predict that enteric viruses could be in biofilms in the distribution system, allowing them to defend against adverse conditions, and eventually effect the human health in natural drinking water [40].

In developed societies this is a reality also (not only in undeveloped regions), with economic losses and public health risks. The infective dose of the virus is influence by the age, health, immunological and nutritional status of the infected individual [124].

Enteric virus infections cause mainly diarrhea and self-limiting gastroenteritis in humans, but it is important to have in mind that some virus cause more severe diseases (e.g. respiratory infections and Hepatitis), with high mortality rates (e.g. aseptic meningitis and encephalitis) in immunocompromised individuals. Some are also thought to be promotors of chronic diseases (myocarditis and insulin-dependent diabetes) [125].

An estimation was made in 1979, between 5 and 18 million people die every year from gastroenteritis, and rotaviruses alone were responsible for over 1 million children dying from diarrhea [126]. Rotaviruses are recognized as the most common viral gastroenteritis agent and norovirus with diarrhea in the infantile and adult population, but in terms of waterborne outbreaks the prior is the most well documented, with rotoviruses and astroviruses only in a few cases [118].

Human adenoviruses (HAdVs) are the second-leading cause of childhood gastroenteritis worldwide. They are very frequent in waters and resistant to disinfection (even UV) [127] mainly causing diseases in the respiratory, ocular and gastrointestinal tracts [128].

Hepatitis A was among the first viruses observed to be transmit by drinking water [4]. Hepatitis cases is generally self-limiting and rarely causing death (may incapacitate patients for months). It has been frequently reported, due to water vehiculation of the virus. Hepatitis E has a higher mortality rate (less number of outbreaks), with special attention to pregnant women that are very susceptible [118].

Emerging waterborne enteric viruses detected in several water sources (from raw to sewage) belong to the families: Caliciviridae (norovirus), Picornaviridae (enterovirus and Hepatitis A virus) and Adenoviridae (adenovirus). Potentially emerging waterborne pathogens are: Hepatitis E virus, the viral agent of avian influenza, coronavirus, polyomavirus, picobirnavirus, and papillomavirus [120].

To detect virus in water the principal execution steps are: sampling, concentration, decontamination/removal of inhibitors, and specific virus access. Sampling procedures are well described [129] but the most important step is concentration that reduce the volume to test allowing the detection of the low numbers of virus particles [116].

There are several factors (water properties) that influence the rates of recover: the pH, conductivity, turbidity, presence of solids in suspension and organic acids [130].

Virus can be detected by cell culture (cytopathic effects) or by molecular amplification techniques [130]. The methods differ in the detection/enumeration step. Ones deal with the viral

infectivity (e.g. viral plaque assay and immunofluorescence foci assay), others with the viral nucleic acid and protein (e.g. qPCR, ELISA) and finally there are the ones that directly count the viral particles (e.g. transmission electron microscopy) [131].

Human adenoviruses are generally a marker drinking water contamination due to their know relation with water outbreaks. Real-time quantitative PCR (qPCR) is a molecular technique with high speed, sensitivity, reproducibility and minimization of contamination [128]. It was used to analyse environment water and water from supplies in Florianópolis, Santa Catarina Island, Brazil, and was demonstrated that HAdV can be efficiently use [127].

Not all the virus can be well detected by plaque assay (absence of a cytopathic effect) or there is not yet available cell culture systems (or grow slowly), while polymerase chain reaction (PCR) alone does not discern between infectious and non-infectious viral particles [130]. The development of a cell-culture assay integrated with a molecular assay (e.g. RT-PCR) could allow the rapid detection of viable viruses [132].

Since the virus presence is a health risk and a lead to economic losses (closures of the water spaces), the improvement of the methods is always to investigators an objective present. [116] Sadly, only using in an unquestioning way the bacterial indicators to infer a fecal contamination (*E. coli* and *Enterococcus* spp.) and to predict viruses could be unreliable, in the understanding of some authors [124].

The occurrence of viral outbreaks demonstrates that the standards based only in coliforms are inadequate to predict the virological quality of water. The reasons usually appointed behind the doubts are: indicator bacteria are more sensitive to inactivation; can have a nonexclusive fecal source (not identify sometimes); low correlation with the presence of pathogens, especially if viruses are in low concentrations [133].

1.4.3.1. Bacteriophages

Direct detection of human enteric viruses (enteroviruses, adenoviruses, noroviruses) may indicate an evidence of human feces presence, but it may be difficult, expensive, and due to some virus that have an intermittent excretion, their absence may correspond to a false negative contamination [74].

It was found a microorganism with characteristics to serve as an indicator of enteric species of viruses, as in bacteria, to obtain a correlation between their presence and the quality of the water. They are called Bacteriophages (phages of bacteria), a group that infect and replicate in bacteria (coliphages if specific to coliform bacteria). They have similar properties and equal, fundamental, characteristics to human pathogenic viruses. They were first though, when discover, to prevention and treatment of bacterial disease (not successfully), but this resemblance with virus allowed biological and medical studies. Their presence reflect viruses metabolism or activities (reproduction was discover this way). They are easily and rapidly cultivated in laboratories (a huge advantage), without huge investments or advance equipment, and detected with simple methodologies [134].

Some bacteriophages can contaminate bacterial cell cultures, if not well carefully eliminated (causing long-lasting consequences). A review about this, give a personal example in their laboratory experience and reveal how some bacteriophages can affect the bacteria fermentation production, inhibit bacterial growth due to lysis of cells [135].

Their role to assess the resistance of human viruses to water treatment, disinfection processes, predict the presence of pathogenic viruses and fecal contamination, is their fundamental function.

Three principal bacteriophages groups, generally found in the gastrointestinal tract, are the F-specific phages, phages that infect *Bacteroides fragilis* and somatic coliphages.

F-specific phages have single-stranded genetic material, infect host bacteria via the F+ pilus and are divide between F-RNA phages (infect through the sides of the pilus) and F-DNA phages (infect through the tip).

A serological classification is used to distinguish F-RNA phages, the group with more potential as an indicator of water contamination, and molecular probes have been already created to all of them. Serotypes II and III have a frequent correlation with human feces (reflect a human pollution), while serotypes I and IV are related with animal feces. The problem with the various subgroups of F-RNA phage is their survival rates that do not comply with viruses; this can confuse the results obtained [74].

Bacteroides spp. species is commonly found in the human gastrointestinal tract (even in larger numbers that coliforms), especially *Bacteroides fragilis.* They are Gram-negative, obligate anaerobic bacteria, do not support the environmental oxygen levels (low rate of survival outside the host) and do not produce spores. These characteristics makes them a possible good indicator of fecal contamination [136].

The bacteriophages of this genus have more advantages, they usually do not replicate outside the host gut and resist to environment adverse (comparable to coliphages). The disadvantages are the more expensive and complex plaque assays, doubts about the *Bacteroides* spp. geographical stability (is crucial to isolate the host specie from a similar place as the sample) [137]. *B. fragilis* also depends on antibiotics supplements and anaerobic condition to grow in culture medium [134].

The somatic phages is the bacteriophage more studied and describe in water samples. They are named coliphages, when they infect *E. coli* (and related species). They are release by humans and other warm-blood animals in feces in huge numbers and is possible to detect them by simple, inexpensive and rapid techniques.

Correlation between enteric viruses and coliphages are always under scrutinium. The presence of enteric viruses was revealed sometimes even with negative results to coliforms [134]. Other results suggest that the absence of coliphages is a good indicator of the absence of enteric viruses, and they may even have a better correlation that bacteria [138].

There are some limitations to the approval of coliphages as a routine test for water quality. Until now: is lacking extensive field testing, is lacking to correlation with a disease occurrence and a stable host [78]. Is hard to find one indicator to predict the entire pathogenic viruses or one to apply in all the conditions, like on bacteria, but the detection of coliphages is being standardised internationally [139].

Studies to find the most useful indicator is an ongoing process and for now, bacteriophages are consider a useful resource as indicators of water quality, complementing the information’s from bacterial indicators [140].

1.4.4. Protozoa

Protozoa are eukaryotic organism with independent individual cells (structurally and functionally). Even species that form colonies do not have a multicellular organization, but have organelles and membranes. Few of them can be seen at the naked eye, nevertheless most of them are microscopic organisms, able to multiply by either asexual division or sexual reproduction [141].

Protozoan parasites are unable to live in an exterior habitat, freely in an environment. They depend on a host (a susceptible organism) to obtain protection and nutrition. They begin their stage as a feeding trophozoites (found intracellularly or extracellularly), within a host, which is fundamental to their parasitic activities. It has the drawback of not being very resistant to the external environment. They are in a constant and fast transition from host to host due to this, using the following strategies: 1) direct; 2) fecal-oral (water); 3) vector-borne (mosquito bite); 4) and predator-prey transmission [142].

They may have no danger to human health and others mammals depending on the protozoa parasite , or else cause some kind of disease/sickness: affect the respiratory tract, central nervous and commonly causing symptoms in the intestines (diarrheia, not deadly) [143]. A report by WHO, in 1998, attributed one-third of all deaths due to parasites activities (about 1.5–2.7 million people die from malaria each year) [144].

The already lack of treated drinking water and sanitations conditions is the biggest promotor of deaths, and an everyday challenge that some part of the world population face. The protozoan parasites can affect millions of individuals without any chance of avoiding them. The three major waterborne protozoan diseases are cryptosporidiosis (e.g. *Cryptosporidium* spp.), giardiasis (e.g. *Giardia duodenalis*) and amoebiasis (e.g. *Entamoeba histolytica*) [33].

People can be induced to think that this is once again a microbiological problem of the less developed societies, but surveillance of drinking water is an international duty to ensure healthy water supplies. The outbreak from Milwauke (United States), in 1993, where an estimated 400 000 individuals suffered from gastrointestinal symptoms due to *Cryptosporidium* spp., is an example of the potential of a protozoan parasite to affect a large number of persons (previous refer) [145].

Giardia spp. and *Cryptosporidium* spp., in the U.S water supply, were appointed as the principal human health risks, both causing gastrointestinal illness [33]. The reported outbreaks are frequently from U.S and the United Kingdom due to their specific surveillance system, which gives a more comprehensive result about the frequency of these protozoan parasites. Others governances should also reflect in relation to this thematic to fully understand the morbidity, mortality, and the value of water treatment systems to control these pathogens [146].

Protection and surveillance against this parasites are further complicate because they produce cysts, a very resistant form (resistant walls) that allows them to survive in adverse conditions. Once in the environment, is just a question of opportunity until the emergence of a route of infection [143].

Parasites like *Giardia* spp. and *Cryptosporidium* spp. produce cysts and oocysts, respectively, facing adverse temperatures and chemical products (e.g. chloride). It starts the growing stage when someone ingests these (oo)cysts. This cycle is responsible for the degradation of human cells and starts the first's symptoms of an infection. In theory, a single (oo)cyst can be enough to multiply and cause symptoms, and *Cryptosporidium* spp. can expel 10^8-10^9 oocysts in feces for 50 days after the diarrhea disappear [147].

The higher temperatures and lack of sanitation, in tropical areas, increases the chance of parasites infections. Malaria is the principal human disease (caused by species of *Plasmodium* spp.) [148]. Infections with *Entamoeba histolytica* (cause amoebic colitis and liver abscesses) are decreasing and is the second most frequent cause of parasitic death (estimated to infect one tenth of the worlds' population or 500 million people) [148].

The most frequent protozoan parasite worldwide is *Giardia duodenalis* (syn. *G.lamblia*, *G. intestinalis*), which causes diarrhoea in 200 million individuals (generally are asymptomatic) with a prevalence of 2–5% in developed countries and 20–30% in developing countries [33]. *Giardia* spp. infection, named giardiasis, has the follow symptoms: dehydration, weight loss, diarrhea, abdominal cramps and fatigue.

An infection by *Cryptosporidium* spp. (cryptosporidiosis) cause: stomach cramps or pain, dehydration, nausea, vomiting, fever, weight loss and death. The total burden of disease still hard to calculate and predict. This microorganism have a ubiquitous distribution. It may cause infection both in humans and animals (throughout fecal-oral route, zoonotic or via contaminated water or food) and have a high environmental contamination (increase the chance of waterborne transmission). Moreover, it has a low-infectious dose (10–30 oocysts), with oocysts environmentally robust and insensitive to the normal disinfectants [147].

It is important to alert that they act also as vectors for intracellular bacteria (between amoebae and bacteria like *Vibrio cholerae*) [149]. This relation is recognized and found in aquatic environments. Free-living amoebae (FLA) are eukaryotic cells from different genera and are an example of opportunistic pathogens. Most bacteria are destroyed inside the FLA host, while some can retain their functions and grow. E.g. *Legionella pneumophila* [150]. The genus *Acanthamoeba* is known for providing a habitat to bacterial growth, allowing their resistance to water treatments and increasing the risk of human illness [149].

The parasites that have a negative impact are *Cryptosporidium* spp. and *Giardia* spp., in terms of drinking water production, due to their ubiquitous distribution, infection potential and survival in water. The frequent routine treatments are, in general, enough to remove them in a safe manner; coagulation/flocculation, sedimentation, filtration and disinfection [33]. Trophozoites are more susceptible that (oo)cysts to the chemicals at disposition. This priors persist even better than most enteric bacteria and viruses. If there are incorrect chemicals treatments and filtration processes, the potential to oocysts infect is exuberate [151].

Promoting efficient chemical treatment, routine monitoring of the systems (multi-barrier methods), professional technicians and the use of ultraviolet irradiation or ozone (examples of others disinfectants available), is possible to inactivate waterborne *Cryptosporidium* spp. and *Giardia* spp. (oo)cysts [146].

Some bacteria may serve as an indicator to give an idea of the parasites present in a water, without assessing each of them individually. Generally *E. coli* is use to predict their presence. *E. coli* is applicable in most circumstances, but in some cases it cannot give a good result because parasites are more resistance than bacteria to the treatments and even when the values for coliform indicator were normal they have occurred [152].

There is not a perfect biological indicator to assess this problem. Is possible to ensure the correct removal of the pathogens, by examining the parameters of a treatment procedure (biological, chemical and physical) [78]. These complications are the reason behind the introduction of some legislation to

monitor the protozoa parasites by the UK Government (2000) with the proposal of a new European directive [153]. It has created the precedent of directly detecting a pathogen, instead of relying in a bacterial indicator for analysing potable water quality. But notes: "*it is unlikely that the coliform standard will be replaced as the primary routine microbiological test of water in the next 20 years".*

Cryptosporidium spp. oocysts from the different species are morphologically similar, so the correct understanding of the species and genotypes that are present, influence the quality of the water management. Protozoa species are so specific to a host, that only detecting the already evident human pathogens is a faster and efficient analyse [154].

The standardized methods to identify *Cryptosporidium* spp. oocysts in environmental samples is frequently the U.S. Environmental Protection Agency method 1622 for *Cryptosporidium* spp. (there are equivalents in other countries like UK) and method 1623 (for both *Cryptosporidium* spp. and *Giardia* spp.). The method 1622 consists in concentration of oocysts by filtration, isolation by immunomagnetic separation (IMS), staining with a fluorescent antibody and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), microscopic detection and enumeration of the stained oocysts [155].

Disadvantages found by some authors are that: this method identifies only the genus *Cryptosporidium*, not making a distinction between species, or the viability/infectivity of detected oocyst, ; and low number of oocysts present in water samples may give a false negative if only one sample is analyse [151]. The current methodologies are limited to perform a correct and routine search. They are time-consuming, the large volume of water that needs to be use helps to confirm the lack of sensitivity of the method and proves the difficulty to detect oocysts [156].

Protozoa represent a group of organism were the modern molecular techniques could overcome the problems present in the conventional methodology [157]. It requires a small volume of water and are commonly more sensitive. Other advantages can be the identification to the level of specie (important to know when dealing with a pathogen or not) that improve the knowledge about the diversity in the sample [141].

Molecular approaches to water tests could overcome some limitations from the 1622/1623 method, especially the species identification (between pathogenic and non-pathogenic) and it allow to assess the source of contamination [154]. It may not only allow their detection, but also provides more data to correlate with values of indicator bacteria giving a more robust knowledge. E.g. in a study using quantitative polymerase chain reaction (qPCR) tests for *Giardia* spp. and *Cryptosporidium* spp. and culture based methods (membrane filtration) for *E. coli* and *Enterococcus* spp.. Comparisons between water samples collected from Chicago different water sources (raw, lake and wastewater) were made. The associations between indicator density and *Giardia* spp. presence, in this study, were observed more consistently (instead of *Cryptosporidium* spp. presence) and associations between *Enterococcus* spp. and parasites were generally stronger (than *E. coli*) [157].

Next-Gen Sequencers is a technique that has been used to obtain faster and complete genomes proving adequated to improved detection of protozoan parasites. Complete genomes are now available for each of the major waterborne protozoan parasites. E.g. *C. parvum* (Iowa) and *C. hominis* (TU502), *E. histolytica, G. duodenalis*. All genotypes with molecular diagnostic markers are still under

development proving able to discriminate virulence and drug resistance. Comparative genomics and proteomics are other potential solutions for specific detection [33].

The techniques and treatments in use are adequate to reduce waterborne parasite infections, plus bacterial indicators can predict their presence, but more investigations are need. These examples demonstrates how the use of modern technologies can be a future solution to monitoring waters for protozoan parasites, with more timely results, a better correlation between the indicators and parasite presence/density. A combined approach might be possible, with molecular techniques being used as a screening tool on a portion of a water concentrate, followed by microscopic examination when positive results are obtained [141].

1.4.5. Fungi

Fungi are eukaryotic microorganisms, mostly dimorphic, with cellulose, chitin and glucans as the basic component of their cell walls. They hinge on an external organic food source to survive/grow (heterotrophic) and they reproduce primarily by developing spores. They have complex cycles of life, having asexual haploid unicellular cells (anamorphic) and sexual reproduction (teleomorphic) suited to survive and disperse in some unfavourable conditions. They are consider as important decomposers in the environment (together with bacteria) [117].

Fungi are found in fresh and seawater environments, but only recently they have been acknowledged as important inhabitants of extreme aquatic habitats [158] and potable water [159] [159] [160]. Fungal colonization may occur inside the piping, after water caption and treatment for drinking use, making up the supply network (sometimes forming biofilms). Those colonization may enhance post-sanitation risk health due to mycotoxins synthesis in water [160].

Fungi living in aquatic ecosystems have to gain access to organic materials to survive and multiply, developing several characteristics. The sea has different salinities (ecosystems in fresh water are markedly different to those in the ocean), and sometimes the water can be particularly acidic or toxic, constraining Fungi growth.

However, there will always be fewer fungal taxa in freshwater habitats compared to terrestrial ecosystems, due to: the exclusion of most basidiomycetes (e.g., only 10 species of basidiomycetes are referred in seawater), zygomycetes and lichens; the lower species diversity of plant hosts in aquatic habitats, and the physiological constraints of submersion in water. The zygomycetes are mostly absent from aquatic habitats, except when the technique of dilution plating of sediments and water is used [161].

The major inputs come from plant materials in streams, for exogenous fungi, organic matter of terrestrial origin (allochthonous), arriving from leaf fall and wood [162]; photosynthetic production (autochthonous matter) is a minor source [163]. These materials contain a range of endophytes of terrestrial origin that are soon replaced by a characteristic group of hyphomycetes with specialised spores.

The fungi at water are known as “Ingoldian fungi” and like the common terrestrial fungi, include many species that appear to lack specificity to the host plant. They also have a worldwide distribution. “Ingoldian fungi” are aero-aquatic hyphomycetes that are not the only fungal inhabitants of litter [164]. Another group is formed from large coiled or rounded anemophilous conidia.

Zoosporic fungi, belonging to *Chytridiomycetes,* can only disperse in aquatic environments. They are commonly named as watermolds, playing a vital role in the degradation of organic matter [165]. The zoospore requires at least a film of water to move away from the sporangium, being readily trapped from water, soil, or from surfaces where a water film forms transiently (e.g., at the edge of water bodies).

Some *Oomycetes* and *Chytridiomytes* species are pathogenic to fish, crustacean, insects and amphibians [166], but the full influence in the aquatic ecology (of Chytridiomycetes) only now is becoming explore and clear. *Chytridiomycetes* is closely associated with processes of removal of phytoplankton (cyanobacteria and microalgae) in lakes and large water bodies. Thus, they are potential biological control agents of important aquatic toxigenic microbes and indicators of eutrophication.

Several reasons to limit the identification of Fungi and understand their distribution exist: many areas are under-collected (collections of raining forests, boreal and tropical regions are still lacking) [167]; several groups have been studied only recently; the importance of fungal enzymes in biotechnology is evolving [168]; convergent evolution in morphology (may interfere with the understanding about the evolutionary relationships) [169]; and cryptic species may be common in local environmental conditions [170].

The ability to more rapidly identify and report aquatic fungi would be greatly facilitated by the development of advanced illustrated keys and monographs that are released and reviewed the information at that time [171]. A centralized geographical database with accepted names and synonymies and others important details for the Fungi identification [170] would further improve the characterization of newly found fungi. The Fungal Genome Initiative is one organization that is being responsible for sequencing key organisms across the fungal kingdom [172].

Population approaches and molecular techniques can determine the phylogenetic relationships among fungi and cryptic species, and give a better perspective. The huge fungal diversity found and assess in aquatic environments, not though before in both marine and freshwater ecosystems, proves that contrary to terrestrial ecosystem, in water, fungi have been largely overlooked [173].

1.5. Chemical hazards monitoring and water treatments

The knowledge of the microbiological characteristics of the waters is relevant to adjust the most appropriate treatment to be implemented, so that water may be considered fit for use. That necessary characterization is achieved by applying standard tests. That evaluation is crucial to be sure that water has the adequate conditions to be used - not only microbiological analyses are used.

Physic-chemical characteristics are also enforced to ensure the adequate quality. They can be vital events, determinant of the water quality. It is imperative to make sure that the maximum allowed concentrations of those chemicals substances, able to cause an adverse effect in human and animals, are not exceeded in the water.

The global number of chemical hazards that may be vehicle by water is not easy to estimate, although they can be calculated in many thousands of molecules. Globally and grossly they can be classified in different categories: residues of substances used by man (antimicrobial drugs, pesticides, phytopharmaceutical drugs; biocides); environmental contaminants or pollutants (heavy metals); natural toxins or biotoxins (substances excreted by bacteria, cyanobacteria, microalgae, plants); chemicals of

the industrial activities; chemicals from the water treatments and distribution (aluminium) [174]. Many attempts to schedule the chemical hazards had been proposed (Table 1.4)

Some of these substances are considered essential priority chemicals, like: fluoride, arsenic, selenium, nitrate, depending on data concerning adverse effects on health already documented.

Table 1. 4 - Categorization of sources of chemicals in drinking-water (adapted from reference [174])

Source	Examples
Naturally occurring chemicals (including naturally occurring algal toxins)	Rocks and soils, cyanobacteria in surface water
Chemicals from agricultural activities (including pesticides)	Application of manure, fertilizer and pesticides; intensive animal production practices
Chemicals from human settlements (including those used for public health purposes; for example, vector control)	Sewage and waste disposal, urban runoff, fuel leakage
Chemicals from industrial activities	Manufacturing, processing and mining
Chemicals from water treatment and distribution	Water treatment chemicals; corrosion of, and leaching from, storage tanks and pipes

Instead of pursuing each potential hazard that may be present, like the monitoring system in use for microbiological hazards, the monitoring of some physical and chemical characteristics of a particular water source may provide an idea of the most relevant chemical hazards (indicators).

Physical attributes are responsible for the aspect of the water; the color, turbidity, total solids, dissolved solids, suspended solids, odor and taste are examples of recorded properties [175].

Suspended solids and colloidal matter alter the turbidity in water; thus interfering with disinfection, also provides a matrix supporting microorganisms growth [176].

Excess of solved minerals (e.g. iron) and biological sediments (e.g. weed or algae), when present, influence the color of water. The odor and taste depend of the living microorganisms or organic decaying matter - they give a notion about the efficiency of water treatment system [175].

Chemical attributes of water may affect all life forms in contact, due to its toxicity; pH, hardness, chemical parameters, biocides, toxic chemicals and biochemical oxygen demand are generally estimated.

The determination of the pH value is an indicator of the water acidity or alkalinity. A low pH helps chlorination, because most living microorganisms are inhibited, but it may provoke corrosion.

Biochemical oxygen demand is the amount of oxygen needed by microorganisms for stabilization of decomposable organic matter under aerobic conditions. High values of DOB indicate organic pollution and less dissolved oxygen to sustain sub aquatic life.

Conductivity is an indirect measure of the salinity of water.

Typical indicators used in water quality monitoring include nitrogen and phosphorous (markers for the presence of nutrients) and chlorophyll-a (a marker for algal and cyanobacteria blooms).

To purify water (removal of adverse chemicals, biological matter, solids or gases) both physical and chemicals procedures can be used. Physical water purification may involve many steps: sedimentation, decantation, flocculation, filtration, UV irradiation techniques. They are very frequently applied in different combinations, depending on the original characteristics of the waters. The thermals

(boiling water) had been the first of all treatments ever applied and is also the cheapest. It cannot be applied to large volumes of water like those existing in a dam. It is, even nowadays, the most practicable in remote societies without access to potable water - particularly in case of risk of epidemic waterborne diseases (cholera).

Chemical compounds are added with distinguishing reactions planed. Chelating agents to prevent negative effects of hardness and flocculation of solved compounds; oxidizing agents to act as biocides, reducing agents that are added to neutralize oxidizing agents.

These previous procedures are usually enough to remove most of the microorganisms and to ensure that all hazards are excluded through disinfection – are essential and decisive treatments.

Disinfection has been applied for longer times, but only in the nineteenth century the effect of disinfectants (e.g. chlorine) was described and became routine's. Disinfection means the removal, deactivation or killing of pathogenic microorganisms by cell wall disruption (plasmolysis) or enzymatic inactivation. There are physical and chemical disinfectants, which may also have a residual effect (remaining active in the water after disinfection). Oxidizing disinfectants also disaggregates organic matter in the water, causing a lack of nutrients [177] (Fig. 1.4).

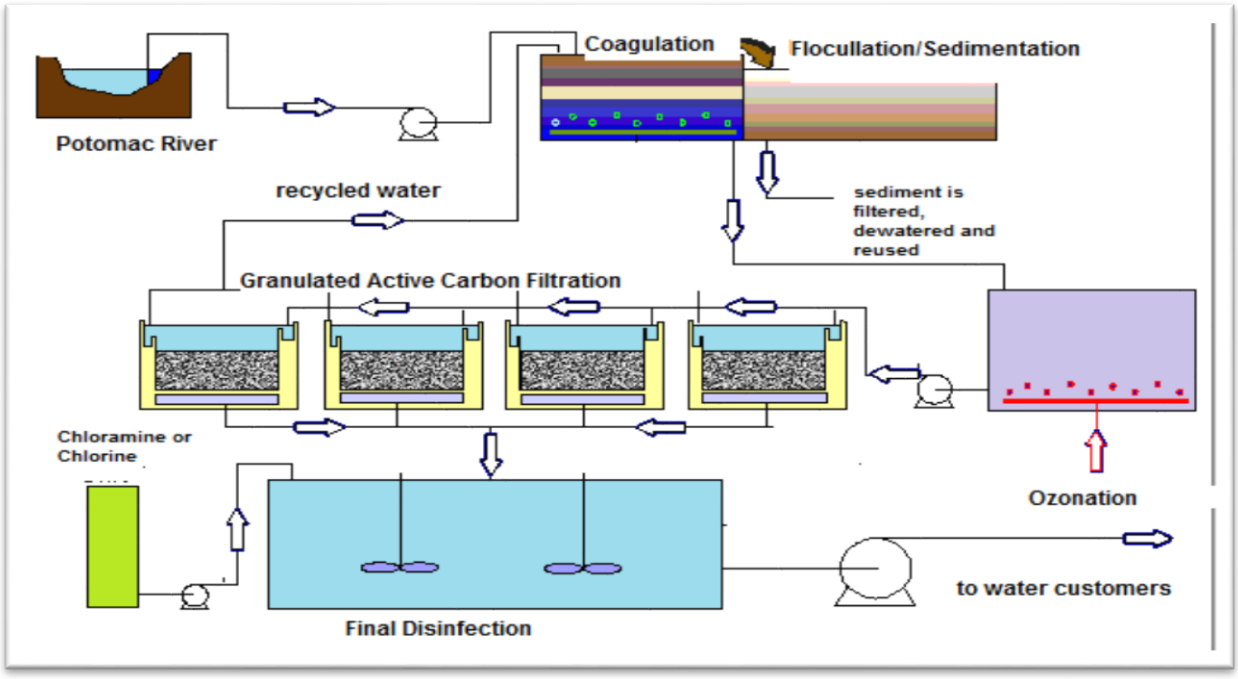


Figure 1. 4 - Diagram of a generic system for water treatment, applied to potable water supply captured from the Potomac River (Corbalis Plant, United States of America)

Halogenated compounds [chlorine (Cl_2), Iodine (I), Bromine (Br_2)], oxidizing compounds (Ozone (O_3)) and quaternary ammonium salts, for chemical disinfection of water, are in use. Ozonation can eliminate abnormal tastes and odors problems, inactivate bacteria or viruses by oxidation and it is one of the most safe procedure in use (higher equipment and operational costs) [178]. Others chemical disinfectants include: metals like copper (Cu), silver (Ag); phenols; alcohols; soaps and detergents; hydrogen peroxide; and several acids and bases. Its application in the treatment of large volumes of water is more unusual.

For physical disinfection of water, electrolysis, ionising radiation and ultraviolet light can be utilized. UV has value in waters susceptible to chemical disinfectants. Nevertheless, physical treatments are safer method of disinfection of water (does not affect the water quality from the chemical point of view) that can destroy bacteria, viruses and other microorganisms in the percentage of 99.97% (alters DNA so that microbes became inapt to multiply) [179].

Chapter II – Evaluation of microbial characteristics of dams´ water in Portugal

2. Specific objectives

Water is fundamental to sustain and promote life, as previously indicated, and should be controlled to assure the maximum quality to their users.

To assure the microbial quality of superficial waters, intended as raw material to produce water for consumption or for recreational purposes is a central task because its usages must be done without risk for human health.

The objective of this work is to perform a preliminary study concerning the microbial characteristic of Portuguese dams´ water. Indicators from water contamination and quality were searched for that,.

Testing enteric indicators in water is generally assumed as relevant to ensure public health protection, avoiding exposure to fecal contaminated waters. Results were also used to verify if the sampling methodology and the season may have some influence in the results. Furthermore, accessory characterization of other relevant microbes was also performed.

Samples obtained using different methodologies were evaluate to verify if there was a significant difference in the values to predict the importance of the place of sampling conditions and the potential disturbances in the values.

2.1. Materials and Methods

The experimental procedures were carried out from September 2014 to March 2015. Selected methods were based on national and international standard procedures, when available.

Microbial analyses are used conventionally to determine water safety. Most frequent parameters used to achieve that goal are the following: enumeration of total cultivable microorganisms, indicators of fecal contaminations (coliforms, *E. coli*, and *Enterococcus* spp.). Other microbial determinations were performed accessorily, namely: search of cyanobacteria and bacteriophages of human enteric bacteria. The limits for the quality for the bacterial indicators followed the current legislation (Decret-Law n.º 306/2007 of August 27) [43].

2.1.1. Sampling

A total of 26 samples of water were collected from 21 Portuguese dams. Samples were collected from September 2014 until March 2015, from 7 different districts of the country and in three regional administrative divisions (NUT 1) (Figure 2.1 and Figure 2.2).

Twenty samples were collected by professional technicians of a laboratory that provides services in water samples collection and analysis. These specialized aseptic recalls of samples were performed on the surface, 0.5m depth of the limnetic zone (epilimnion) in the middle of the dams, using a boat (“professional sampling”). Six water’s samples from Portuguese dams were directly collected by the team work, accessing to the limnic zone of the water column, in the margins of four dams (“direct sampling”).

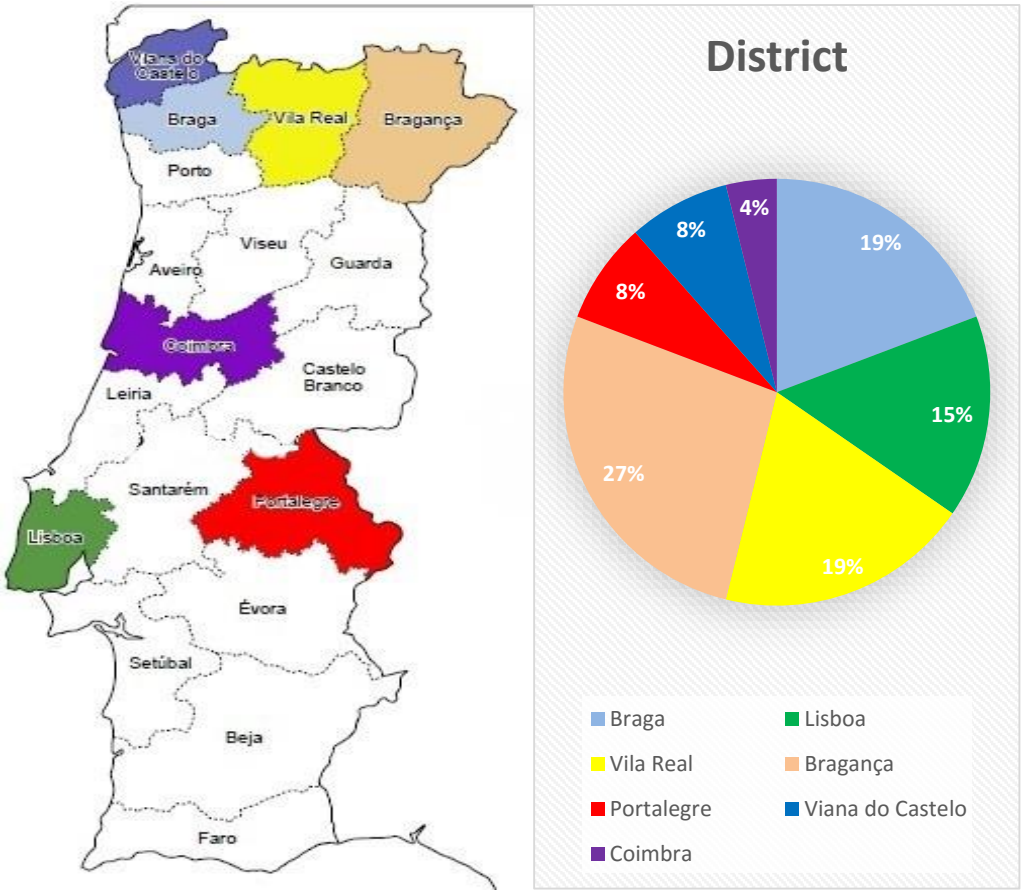


Figure 2. 1 and **Figure 2. 2** - Districts where the sampling occur and the relative frequency associated.

The water samples were collected in sterile Van Dorn glass bottle, having a maximum volume of 1000 mL. They were identified, conditioned in cool boxes and transported to the laboratory maintained under a recommended temperature, <4 ºC. All samples were store in a refrigeration chamber at 3 ºC for a maximum of 5 days, until the analytical procedures were executed.

After collecting the aliquots used for the current microbiological analyses, about 100 mL of water were transferred for another sterile glass bottle to preserve cyanobacteria, and more 20 mL from the former sample to perform the bacteriophages detection.

2.2. Analytical procedures

Six different analytical procedures were executed in terms of microbial determinations: enumeration of total cultivable aerobic microorganisms at 22 ºC and 37 ºC, enumeration of total coliforms, enumeration of *E. coli*, enumeration of *Enterococcus* spp., detection of human enteric bacteriophages, detection and identification of cyanobacteria. A small number of samples were preliminaries tested for fungi search, but its results will not be discussed in this work.

2.2.1. Enumeration of total cultivable aerobic microorganisms at 22 ºC and 37 ºC

The enumerations were performed using a pour plate technique, seeking to determine the number of aerobic microorganisms present in the sample. Pour plates are prepared mixing an aliquot of the water sample with molten culture media Yeast Extract Agar in Petri dishes 9 mm diameter,

followed by incubations at different temperatures. All visible colonies are counted and the result was expressed as “colony forming units” (CFU)/ ml, after incubation.

The analytic procedure was performed following a protocol base in the ISO 6222: concerning “Water quality” – Enumeration of cultivable microorganisms – colony count by inoculation in a nutrient agar culture medium. The first step was the registration of the details concerning the sample identity: source or origin, date of collection; date of analytic procedure starting; the dilution expected to contain between 30 and 300 CFU/ mL and the dilution volume of the plated sample (always 1.0 mL).

All samples were stored at refrigeration temperatures and were adequately homogenized before processing. The media, Yeast Extract Agar, once molten, was equilibrated at 50 ± 1 °C in a water bath (Memmert®) and kept there until required. No more than 2 hours from the time the agar reaches 50 ± 1 °C. It was inoculate a labelled empty “Petri dish” with the aliquot of the diluted specimen (1 mL), in duplicate, starting with the most dilute sample solution.

The culture agar, in the flask (still molten), was added to the center of a Petri dish within 20 minutes of dispensing the 1 mL / sample / dilution. The dishes were then cover with lids and gently mixed, tilting and swirling the dish gently (‘hand plate pouring’), clockwise and anti-clockwise circular movements for approximately 10 seconds to ensure that the culture medium were thoroughly mixed and the medium covers the plate evenly. The agar was undisturbed for about 10 minutes to solidify completely, and incubated in an inverted position. One serial of the “Petri dishes” (two / dilution) were incubate at 22 ± 2 °C for 68 ± 4 hours and the other Petri-dish at 36 ± 2 °C for 44 ± 4 hours.

All the colonies were counted, after the incubation time, using a magnifying colony counter. Were then recorded, and calculated as CFU/ 1 mL, using the following formula:

$$\text{CFU/ 1 mL} = (\text{number of CFU/ plate} \times \text{dilution factor})/ \text{aliquot}$$

The colonies were counted and results were validated when the number of colonies per Petri dish was inferior to 300. If the count were greater than this value it was count from Petri dish containing the most diluted aliquot, with 10 to 300 colonies. If all “Petri dishes” showed more than 300 colonies, the result was recorded at greater than 300 at the highest dilution.

When no dilutions were performed counts greater than 300 per “Petri dish”, has been recorded as >300 CFU/ mL.

2.2.2. Enumeration of total coliforms and *Escherichia coli*

Coliforms are a functional group of Gram - negative bacteria that ferment lactose, producing acid and gas, in the presence of bile salts (2%) and that are oxidase negative. Belonging to coliforms, the bacteria *Escherichia coli* can release indole from tryptophan at 44.0 ± 0.5 ºC in 21 ± 3 hours.

An analytical procedure based on ISO 9308-1 norm, to quantify coliforms and *E. coli* in samples, relative to “Water quality – Detection and enumeration of *E. coli* and coliform bacteria”, was followed. A filtration technique was used. Firstly, “Petri dishes” holding 20 mL of Tergitol-7 Agar, (Oxoid, CM0793) were removed from its package, labelled on their underside (number of the sample date, targeted microorganisms and dilution in use) to prevent errors. Water samples were kept at environmental temperature and adequately mixed before testing.

Volumes of 100 mL of the sample, or decimal dilutions (10 mL, 1 mL) were filtrated through a sterile nitrocellulose membrane (Porosity: 0.45 µm; Diameter: 47 mm; Pall®). When the sample volume was inferior to 100 mL (diluted), it was added 10 mL of sterile physiologic solution to a vacuum filter holder (EMD MILLIPORE CORP XF2004710) before applying the vacuum (Millipore-XF54 230 50). This help to obtain a more uniform distribution of bacteria evenly across the entire filter surface.

Sterilized forceps were used to remove the membrane filter from the package. The filter membrane was then centred on the holder base with the grid side up. The filter funnel was placed onto the assembly and fixed. The pouring lip of the sample container was flamed and the sample (or starting with the highest sample dilution to be tested) was poured in the funnel of the filtration unit. The vacuum pump (Millipore-XF54 230 50) was switched on, dispersing the sample evenly over the membrane to prevent the cluster of bacteria. The vacuum was turned off, after filtration. The sample was completely drained through the filter. Sterile forceps were used to remove the membrane from the holder and place on the media surface (Tergitol-7 Agar, Oxoid, CM0793), ensuring that there were no air bubbles between the filter and the media, in the “Petri dish”.

The procedure was repeated until all dilutions for one sample were complete. The filtration unit was washed for each sample to prevent contamination. Within the same sample, starting from the higher dilution, there was no need to wash.

The “Petri dishes” were incubated in an inverted position at 37 ºC in an air incubators (Memmert - UF55).

The presence of lactose in the culture medium promotes the formation of acid, a characteristic of the coliform group, which is detected by the color change of the media. Typical colonies have a yellow color with, or not, an orange center (*E. coli* and *Citrobacter* spp.); with alteration in the culture media (also yellow). These colonies are considered “lactose – positive”. Colonies with other colors can also appear; *Enterobacter* spp. present a red or yellow color, no color in the centre and yellow in the middle. Colonies that do not ferment lactose are red or purple and change the color of the medium to blue (alkalization).

All the typical colonies were subject to confirmation, at least 10 colonies per plate. The preliminary biochemical characterization was made, detecting the enzyme oxidase and indole formation. Colonies were isolated to a non-selective medium, Tryptone Bile Agar (TBA) (Oxoid, CM0595), and to tubes with Peptone water (PW) (Oxoid, CM0009). The inoculated media were incubated at 37.0 ± 2.0 ºC for 21 ± 3 hours and the tubes with PW at 44.5 ± 0.5 ºC for 21 ± 3 hours.

The oxidase test was performed with colonies in TBA, after culture incubation. This test evaluates the presence of the enzyme oxidase, which reduces oxygen along the electron transport chain. The test was realized by pouring two or three drops of the oxidase reagent [extemporaneously prepared, a 10% solution of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride in water (Difco - 0329-15)] over a portion of the isolated colony obtained in the medium TBA (on a filter paper) with a plastic loop.

When the spot turned to a deep blue-purple color, after 30 seconds, it was considered a positive reaction; oxidation of the reagent by the oxidase enzyme. If the color do not change, is a negative reaction; *E. coli* is an oxidase – negative bacteria.

The indole test was also used, to distinguish *E. coli* from the others coliforms[69]. This specie can release indole from tryptophan (decarboxylation) at 44.5 ºC. The reaction is revealed using some drops of Kovacs reagent added to an overnight culture of the isolated. The appearance of a red layer ring in the surface of the tube culture reveals the ability of the bacteria to release indole from tryptophan. Typically colored colonies, oxidase-negative were considered coliforms; typical colored colonies, oxidase-negative and indole-positive at 44.5 ºC, were considered *E. coli*.

All the colonies, after confirmation, were counted, calculated and expressed as CFU/ 100 mL coliforms and CFU/ 100 mL *E. coli* with the following formula:

CFU/ 100 mL = (number of CFU/ plate x dilution factor)/ aliquot

2.2.3. Enumeration of *Enterococcus* spp.

Intestinal *Enterococci* are Gam-positive bacteria, coccus-shaped, which are able to reduce 2,3,5-triphenyltetrazolium chloride to formazan and to hydrolyse aesculin at 44 °C on a culture media like Bile Esculin Agar (BBL - 299068).

Enterococcus spp. is regarded as indicator of fecal contamination, in the context of microbial water examination [180]. The method chosen (describe forward) is especially intended for examination of drinking water, water from swimming pools and other disinfected or clean waters.

To detect and quantity *Enterococcus* spp., in water samples, an analytical procedure base on the ISO 7899-2, concerning “Water quality: Detection and enumeration of intestinal *Enterococci*” was used.

A membrane filter, with porosity: 0.45 µm and diameter 47 mm (Pall®), was placed using sterilized forceps, with the grid side up, on a support surface of a vacuum filter holder (EMD MILLIPORE CORP XF2004710). The forceps were sterilized by flame, and cooled before use. The necessary dilutions were prepared to obtain an adequate dilution work aliquot of the water sample. The sample was homogenized by agitation for some seconds, and put into the filtration funnel. Vacuum was applied to filter the sample using a pump (Millipore-XF54 230 50). When the volume of the test sample was inferior to 100 mL (diluted), 10 mL of sterile water was added to the filter funnel before applying the vacuum to ensure a more uniform distribution of the microbes. Vacuum was released when the filter became dried and the lift was took from the funnel top. The membrane filter was transferred to previously prepared agar plates, using sterilized forceps, having “Slanetz & Bartley Agar” (Liofilchen, 610134). The filter was placed, in a position with the grid side up, on the surface of the agar. Air trapped under the filter was checked and it was made sure that the entire filter touches the agar. The “Petri dish” were inverted and incubate at 36.0 ± 2.0 ºC for 44 ± 4 hours.

The typical colonies in this medium are small and exhibit a reddish, brown or pink dark color. They were enumerated when typical morphologies of the colonies were observed, and a subsequent confirmation procedure was executed. The specific identification of the genus *Enterococcus* was accomplished incubating picked colonies to “Bile esculin agar (Scharlau® 064-TA0102) at 44.5 ± 0.5 ºC for 2 hours. This bacteria genus hydrolyses the esculin, developing esculetin and dextrose. The esculetin reacts with the ferric citrate present in the media, developing a complex black color.

The final account was immediately made for colonies with characteristic morphologies and recorded as belonging to the genus *Enterococcus*. After confirmation, the results were expressed in CFU/ 100 mL using the following calculation formula:

CFU/ 100 mL = (number of CFU/ plate x dilution factor)/ aliquot

2.2.4. Detection of Cyanobacteria

Cyanobacteria are a group of autotrophic microorganisms, having, some of them, potential to produce toxins, hazardous to human health and all life. Their presence in the superficial waters affects its quality [93]. Detection and quantification of these bacteria, like the typical analyses of phytoplankton, are a very challengeable task, since it demands specialized skills. It is widely recommended to observe the samples *in vivo*, to avoid the destruction of the species, or changes in their morphology, that are the base of their identification.

In the context of the present work, an adapted operating procedure was developed and applied. With it, the preservation of cyanobacteria in the water sample was judged, aiming subsequent identification (*in vivo*), using microscopic techniques.

After the microbiological tests previously described, approximately 200 mL were removed to new sterilized flasks, from the water samples of each dam (flasks of 1000 mL), without being full. These aliquots were aseptically transferred, and were kept in incubation at room temperature in the laboratory facing the daylight. Shortly, small cylinders of a previously prepared culture media (modified “BG-13 Agar”) were put inside, aiming to sustain the cyanobacteria presence. This medium has been used in isolation and growth of cyanobacteria [181].

An aliquot of each sample were centrifuge at low rotations (2000g) for 20 minutes, to concentrate the samples and obtain a primary notion concerning the presence of those microorganisms.

Different colorations techniques were assayed:

- Victoria Blue with Giemsa, results to differentiate the genus.
- Malachite Green with Lugol.
- The application of a single drop of safranine, a simple procedure, allowed the visualization in every sample.

To detect and identify algae and cyanobacteria, till the genus taxa, optic microscopic visualizations were executed using a maximum magnification of 400x. The images were caught with a digital camera and stored in a computerized system. Typical micro morphologies of cyanobacteria genera were identified comparing with taxonomic keys generally recognized [182].

Other confirmations were performed accessorily. Some guides were used for that, including digital libraries of images assessable by internet [182 –188].

The relative frequency of toxigenic genera was established in each positive sample, because cyanobacteria are more relevant when associate with toxins productions [190]. Cell enumeration and cyanotoxins quantifications were not tried, although some preliminary cultures were essayed using plating account in modified “BG-13 Agar” [181].

2.2.5. Detection of bacteriophages of human enteric bacteria

Bacteriophage, also called phage (or bacterial virus), are a group of viruses that infect bacteria. Most of them have capacity to cause bacteria lyses. Thousands of varieties of phage exist, each of which may infect strict or a large board of bacteria species host.

Plaque lyses are visible, when formed, on the surface of a host-bacteria layer cultured on a non-selective nutrient medium (Plate count agar, Oxoid - CM 0325). Counting the number of plaques in the higher decimal dilution, where lyses was detected, can be used as a method for quantification of shigaphages [191].

The following procedure was executed: Primarily a 10 mL culture of host cells (*Shigella sonnei* ATCC 25 931) were grown in "Nutrient broth" (Oxoid, CM 0001) at 37 ºC for 24 hours, by sterile transfer.

An aliquot of each water sample (about 20 mL) was added to 20 mL of "Nutrient broth" double concentrated and 1 mL of the culture of the host-bacteria (*Shigella sonnei*). All the ensemble was incubated for 24 hours at 37 ºC. This water sample culture was filtered through 0.45 µm nylon membrane filter (VWR®) (25 mm diameter) with a syringe (Terumo®). Bacteria were retained and the eventual phages were collected in filtrate. About 0.1 mL milliliters of the filtrate was deposited (fresh, after filtrate) on the surface of a host-bacteria uniformly smeared on "Plate Count Agar" (Oxoid - CM 0325). Grow cultures were incubated overnight at appropriate bacterial temperature (37 ºC) for 24 hours in air incubator (Memmert - UF55).

"Plate count agar" (Oxoid CM 0325) was used as the medium for the growth of the host bacteria cultures, propagation and eventual plaque-counting of bacteriophage. The cultures were observed, after incubation, to detect any plaque of lyses.

2.6. Statistical analyses

The results that were obtained for the enumeration of cultivable microorganisms, coliforms, *E. coli* and *Enterococcus* spp. were submitted to different statistical analysis attempting to verify if there were any correlation or variability associable to some exogenous factors: seasonality, sampling procedure, geographic origin of samples.

Data were subjected to statistical tests using a free software environment for statistical computing - "R Project for Statistical Computing" [192]. The most commonly used tests has been: Normalization by "Shapiro.test", "ANOVA", "Kruskal-Wallis" chi-squared, df, "t.test", "Wilcoxon rank sum", "Spearman's rank correlation". For all tests, the p value 0.05 was used.

2.3. Results

2.3.1. Enumeration of cultivable microorganisms

Cultivable microorganisms at 37 ºC were detected in all samples (100%) of water collected from Portuguese dams (Figure 2.3).

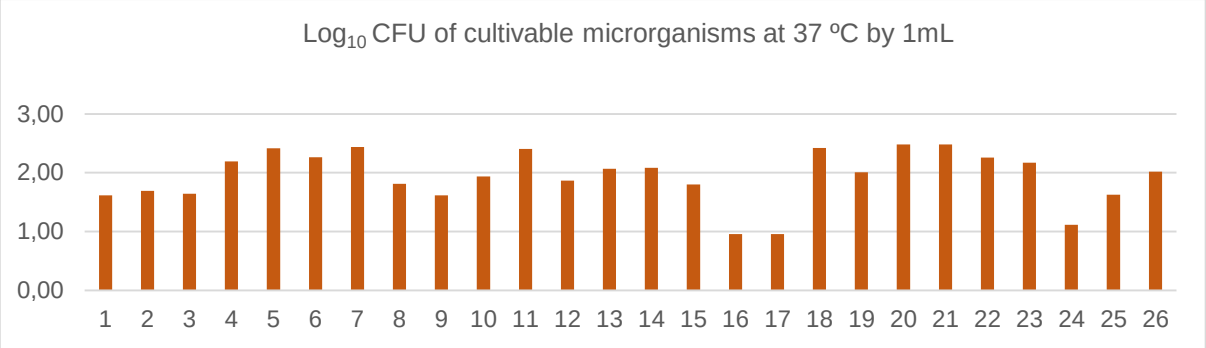


Figure 2. 3 - Enumeration of cultivable microorganisms at 37 ºC in Portuguese dams' water (September 2014 to March 2015) (CFU log_{10} values by 1 mL

The results were grouped following the current legislation (Decret-Law n.º 306/2007 of August 27) [43]. Two of the samples showed general microbial burden superior to 2.48 log_{10}/ 1 ml. Thirteen samples were under the limit 2 log_{10}/ 1 ml (50%) and eleven were between 2 log_{10}/ 1 ml and 2.48 log_{10}/ 1 ml (42.31%) (Table 2.1).

Table 2. 1 - Distribution of cultivable microorganisms at 22 ºC in Portuguese dam waters (September 2014 to March 2015) (CFU log_{10} values by 1 mL)

		CFU log_{10} by 1 mL		
Nº of samples	26	<2	>2 & <2.48	>2.48
Presence	26	13	11	2
Frequency (%)	100	50	42.31	7.69

Legend: CFU - colony forming unities

Cultivable microorganisms at 22 ºC were also detected in all water samples (100%). Eleven of them showed general microbial burden superior to 2.48 log_{10}/ 1 ml (42.31%) (Figure 2.4).

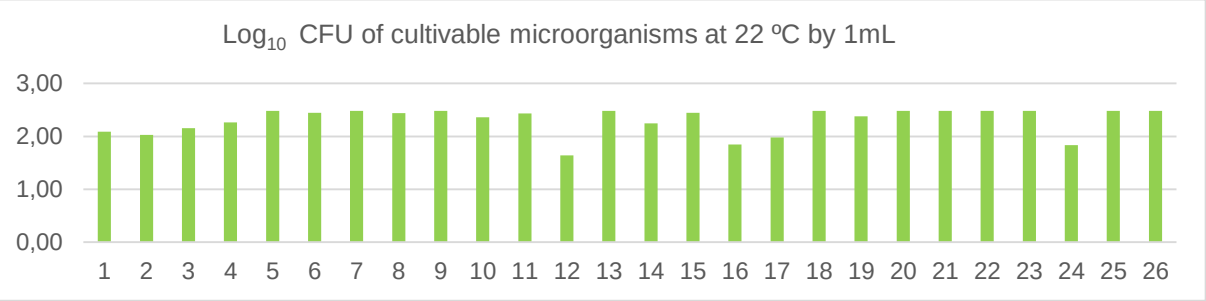


Figure 2. 4 - Enumeration of cultivable microorganisms at 22 ºC in Portuguese dams' water (September 2014 to March 2015) (CFU log_{10} values by 1 mL)

Four samples were under the limit < 2 log_{10}/ 1 ml (15.38%) and eleven were between 2 log_{10}/ 1 ml and 2.48 log_{10}/ 1 ml (42.31%) (Table 2.2).

Table 2. 2 - Distribution of cultivable microorganisms at 22 ºC in Portuguese dam waters (September 2014 to March 2015) (CFU log_{10} values by 1 mL)

	Samples	CFU log_{10} by 1 mL		
Number	26	< 2	> 2 & < 2.48	>2.48
Presence	26	4	11	11
Frequency (%)	100	15.38	42.31	42.31

Legend: CFU - colony forming unities

2.3.1.1. Comparison of cultivable microorganisms at 37 ºC and 22 ºC

The values for cultivable microorganisms at 22 ºC were always superior to those at 37 ºC (Figure 2.5) but inferior in the sample 12.

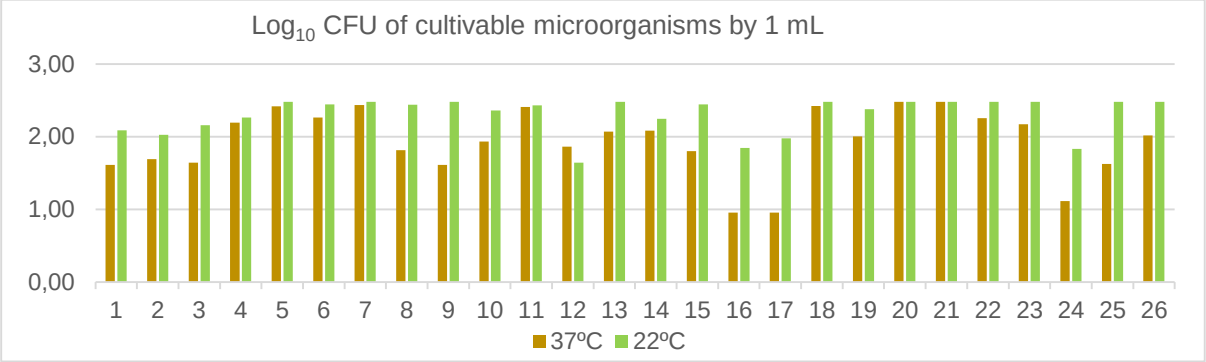


Figure 2. 5 - Enumeration of both cultivable microorganisms at 37 ºC and 22 ºC (CFU log_{10} values by 1 ml) found in Portuguese dams water samples (September 2014 to March 2015)

A positive correlation was found between cultivable microorganisms at 37 ºC and 22 ºC (rho = 0.62, p <0.05).

2.3.2. Enumeration of *E. coli* and total coliforms

Twenty samples were used to calculate both *E. coli* and coliforms burden. Samples 16, 17, 18, 19, 24 did not revealed the presence of *E. coli* (Figure 2.6).

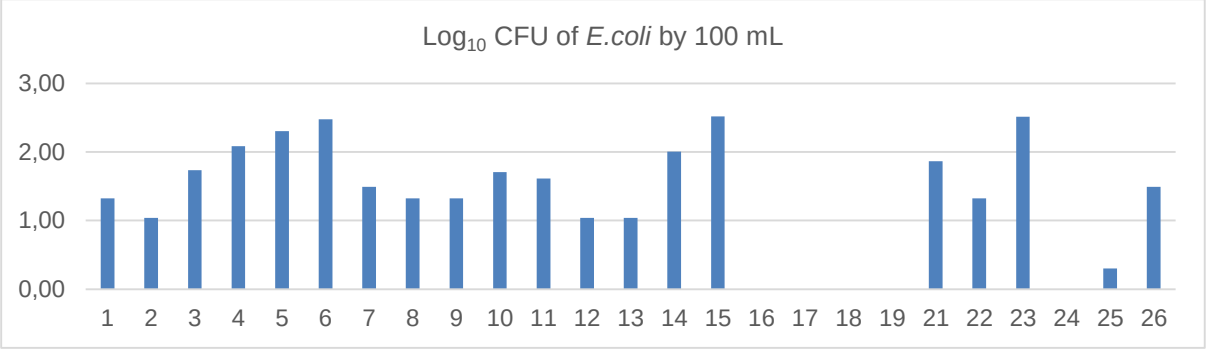


Figure 2. 6 - Enumeration of *E. coli* (CFU log_{10} values by 100 mL) in Portuguese dams' water (September 2014 to March 2015)

The results were grouped following the current legislation (Decret-Law n.º 306/2007 of August 27) [43]. None of the samples contaminated with *E. coli* showed general microbial burden superior to

4.30 log_{10}/ 100 ml. Twelve samples were under the limit 3.30 log_{10}/ 100 ml (46.15%) and thirteen samples were under the 1.30 log_{10}/ 100 ml limit (50.00%) (Table 2.3).

Table 2. 3 - Distribution of *E. coli* (log_{10}) in Portuguese dam water's samples (September 2014 to March 2015)

	Samples	log_{10} *E. coli* by 100 mL		
Number	26	1.30	3.30	4.30
Presence	20	13	12	0
Frequency (%)	76.92	50.00	46.15	0.00

Legend: CFU - colony forming unities

The sample number 18 was negative, concerning to coliforms frequency (Figure 2.7).

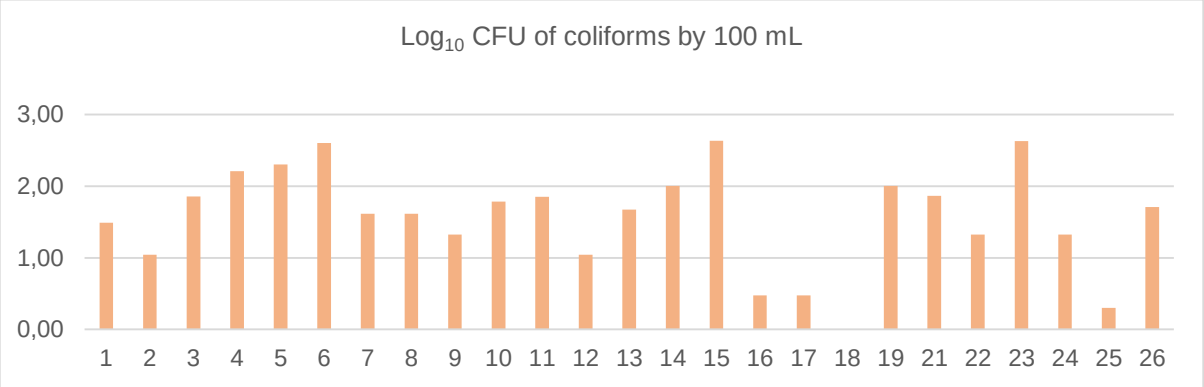


Figure 2. 7 - Enumeration of coliforms (CFU log_{10} values by 100 mL) in Portuguese dams water (September 2014 to March 2015)

Coliforms were detected in 24 water samples (92.31%). None of the samples showed general microbial burden superior 4.70 log_{10}/ 100 ml. Eleven samples were under the limit 3.70 log_{10}/ 100 ml (42.31%) and fourteen samples were under the 1.70 log_{10}/ 100 ml limit (53.85%) (Table 2.4).

Table 2. 4 - Distribution of coliforms (log_{10}) in Portuguese dams' water (September 2014 to March 2015)

	Samples	log_{10} coliforms by 100 mL		
Number	26	1.70	3.70	4.70
Coliforms presence	24	14	11	0
Frequency (%)	92.31	53.85	42.31	0.00

Legend: CFU - colony forming unities

2.3.2.1. Comparison of coliforms and *E. coli*.

Samples number 16, 17, 19 and 24 did not reveal the presence of *E.coli*, while sample number 18 were negative for both *E. coli* and coliforms. Sample number 20 was contaminated with a not calculable number of both group of microorganisms (not conclusive). All the values for coliforms were superior to *E. coli* (presence of unidentified coliforms) or equal (all coliforms were *E.coli* in seven samples) (Figure 2.8).

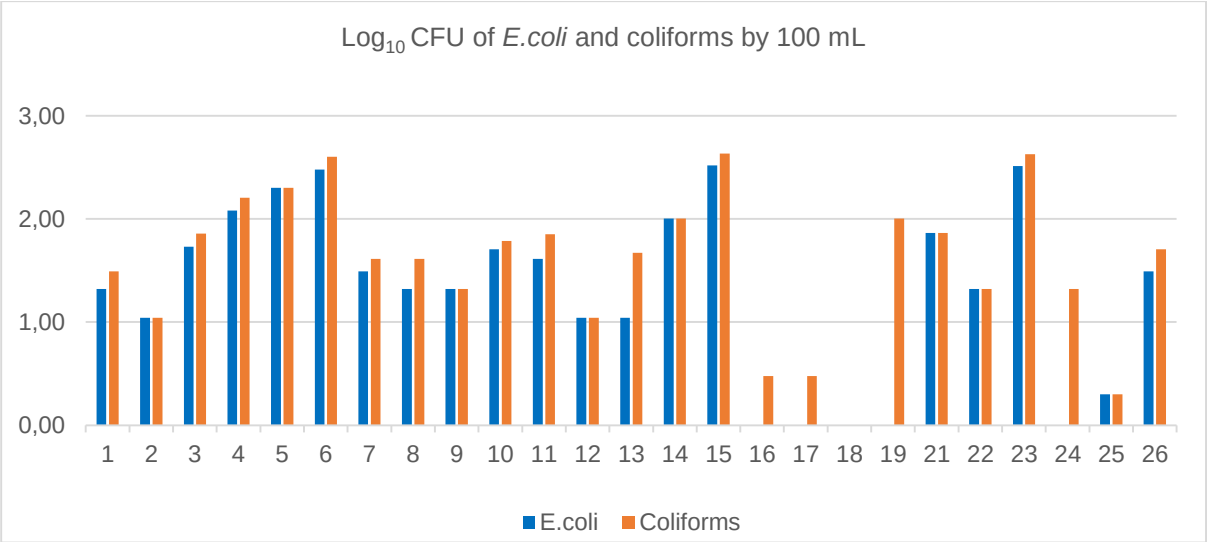


Figure 2. 8 - Enumeration of both *E. coli* and coliforms (CFU log_{10} values by 100 mL) in Portuguese dams' water (September 2014 to March 2015)

A positive correlation was found between them (rho = 0.85, $p < 0.05$).

2.3.3. Enumeration of *Enterococcus spp.*

Samples number 10, 12, 16, 17, 18, 19 and 24 did not revealed the presence of *Enterococcus* spp. in 100 ml of water (Figure 2.9).

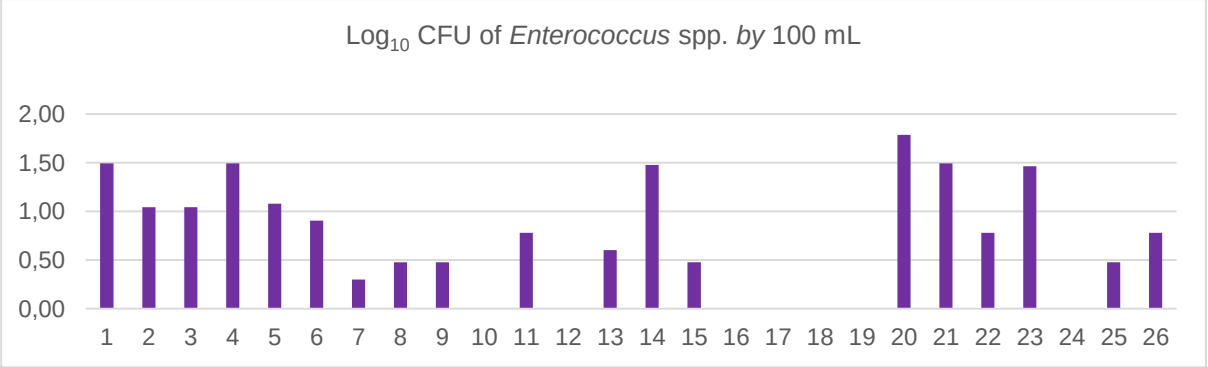


Figure 2. 9 - Enumeration of *Enterococcus* spp. (CFU log_{10} values by 100 ml) in Portuguese dams' water (September 2014 to March 2015)

Enterococcus spp. were detected in 20 water samples (76.92%). The results were grouped following the current legislation (Decret-Law n.º 306/2007 of August 27) [43]. None of them showed general microbial burden superior to 4 log_{10}/100 ml. Six samples were under the limit 3 log_{10}/100 ml (23.08%) and twenty were under the limit 1.30 log_{10}/100 ml (76.92%) (Table 2.5).

Table 2. 5 - Distribution of *Enterococcus* spp. (log_{10}) in Portuguese dams' water (September 2014 to March 2015)

		Log_{10} by 100 mL		
Nº of samples	26	1,30	3	4
Presence	20	20	6	0
Frequency (%)	76.92	76.92	23.08	0.00

2.3.3.1. Comparison of *E. coli* and *Enterococcus spp.*

Concerning the samples number 16, 17, 18, 19 and 24, none of those two indicators were present. In the samples 10 and 12 only *E. coli* were present, while in sample 20 only *Enterococcus* spp. are accounted. Sample 20 revealed an incalculable number of *E.coli*, considering the limits of the methodology that was used. The negative value 1 represents the uncountable of *E. coli* in the sample (figure 2.10).

To evaluate both fecal contamination indicators this analyse was made and was evident from the results that *E. coli* has been always higher than the correspondent for *Enterococcus* spp.. *Enterococcus* spp. was present in more two samples than *E. coli* and its enumeration has been always possible.

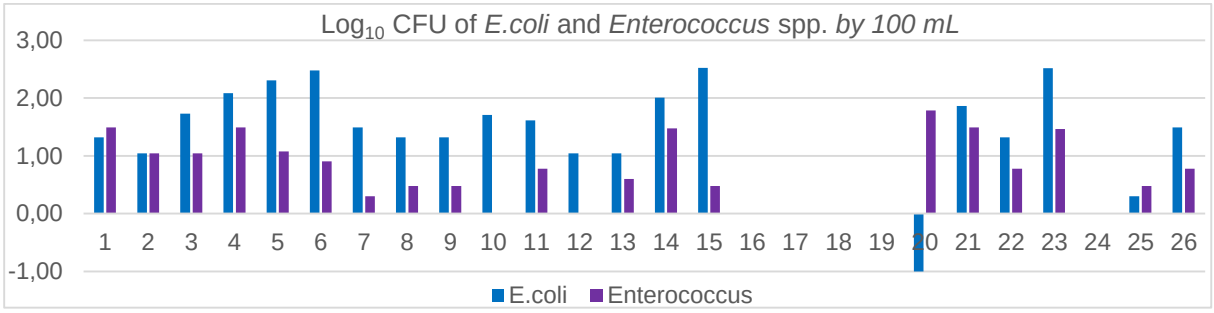


Figure 2. 10 - Enumeration of both *E. coli* and *Enterococcus* spp. (CFU log_{10} by 100 mL) in Portuguese dams' water (September 2014 to March 2015)

A positive correlation was found between both parameters (rho = 0.66, p <0.05).

Coliforms and *Enterococcus* spp. (annex 1) revealed a positive correlation (rho = 0.53, p <0.05).

2.3.4. Comparison of results obtained with "direct sampling" and "professional samples"

The mean values in the groups chosen were higher in the direct sampling, generally, than those obtained from samples collected professionally. The number of culturable microorganisms really showed this tendency. The values for direct samples for cultivable microorganism at 37 ºC and 22 ºC are almost constant, having a mean of 2.34 ± 0.18 CFU/ 1 mL and 2.46 ± 0.04 CFU/ 1 mL respectively, while samples collected professionally had mean values of 1.81 ± 0.43 CFU/ 1 mL and 2.25 ± 0.27 CFU/ 1 mL. (Figure 2.11 and Figure 2.12).

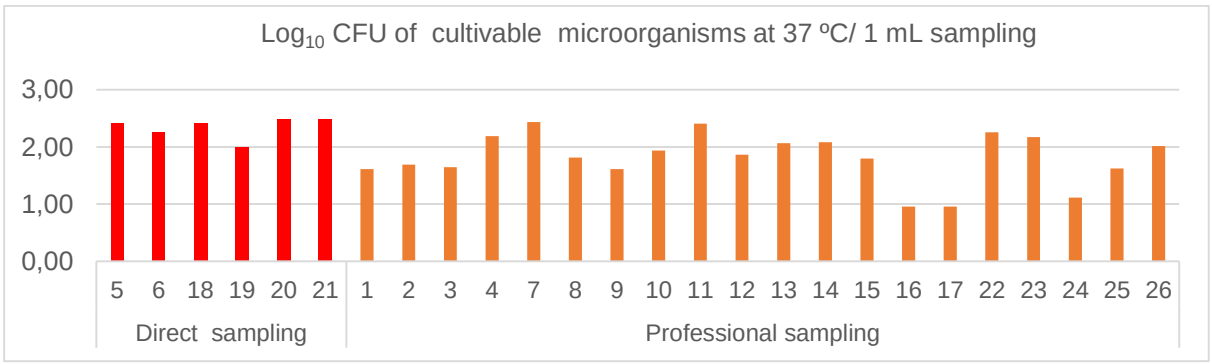


Figure 2. 11 - Comparison of cultivable microorganisms at 37 ºC using two sampling procedures ("direct sampling" and "professional sampling") (CFU log_{10} values by 1 mL)

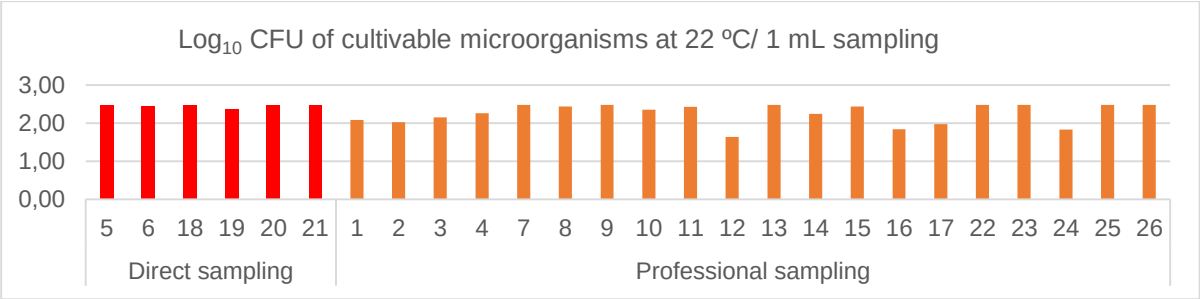


Figure 2. 12 - Comparison of "direct sampling" and samples obtained from a professional laboratory for cultivable microorganisms at 22 ºC (CFU log_{10} values by 1 mL)

The sample number 20 were not included in the calculations for *E. coli* and coliforms due to the fact that the results were uncountable in the used methodology. The mean values for *E. coli* and coliform (annex 2 and 3) were higher, like the value for *Enterococcus* (mean 0.88 ± 0.75 CFU/ 100 mL *versus* 0.64 ± 0.52 CFU/ 100 mL) in the direct samples (Figure 2.13).

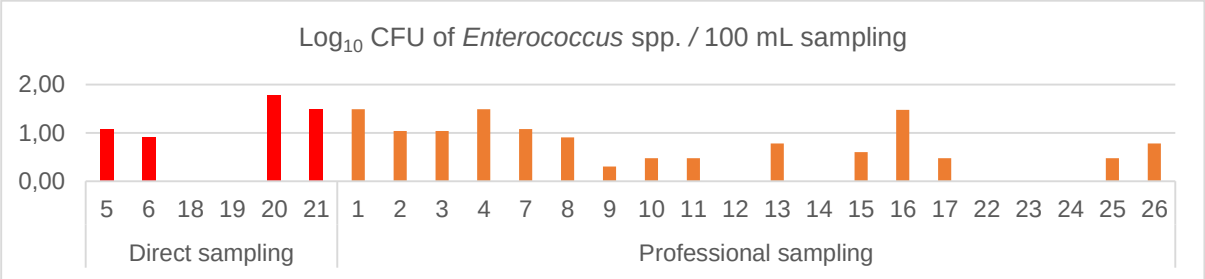


Figure 2. 13 - Comparison of results obtained for *Enterococcus* spp. (CFU log_{10} values by 100 mL) with "direct sampling" and samples collected by a "professional laboratory"

Using statistical analyse, only cultivable microorganisms at 37 ºC showed a correlation with the sampling ($p<0.05$).

2.3.4.1. Comparison of samples collected in two different seasons

The means values obtained in the autumn and winter season were superior in the first group refer to *E. coli* and coliforms (annex 4 and 5). The autumn group revealed inferior to values of the winter group for the means values of *Enterococcus* spp., but with a much clear margin (0.61 ± 0.55 CFU/ 100 mL and 0.97 ± 0.64 CFU/ 100 mL) (Figure 2.14).

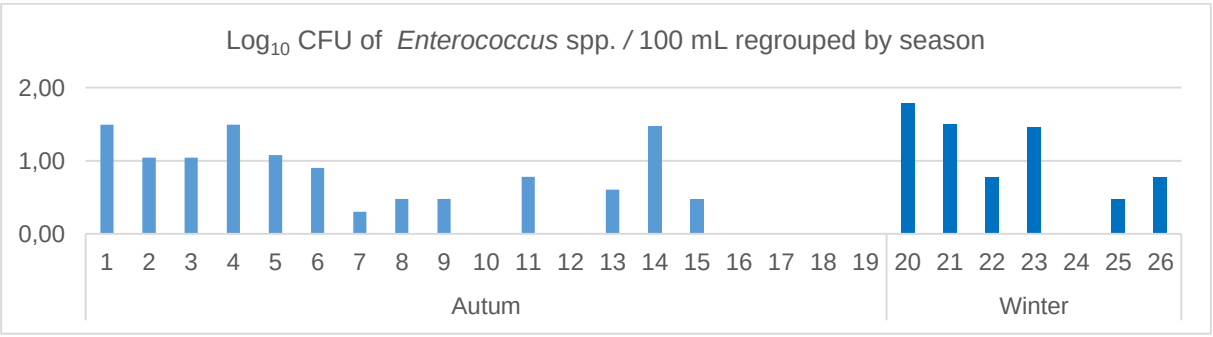


Figure 2. 14 - Comparison of samples from different seasons (CFU log_{10} values by 100 mL)

Cultivable microorganisms for both temperatures showed an increase in the mean value in the winter season (annex 6 and 7). Only cultivable microorganisms at 22 ºC showed a correlation with the season ($p<0.05$).

2.3.4.2. Comparison of samples from the same dam

Some samples were collected from the same dam at different times, and were analysed to verify if the values were maintain or not (Figures 2.15 and 2.16). This is especially important in the case of the cultivable microorganisms, which a constant value is a signal of steady conditions. An alteration could reveal a perturbation, and a possible health risk.

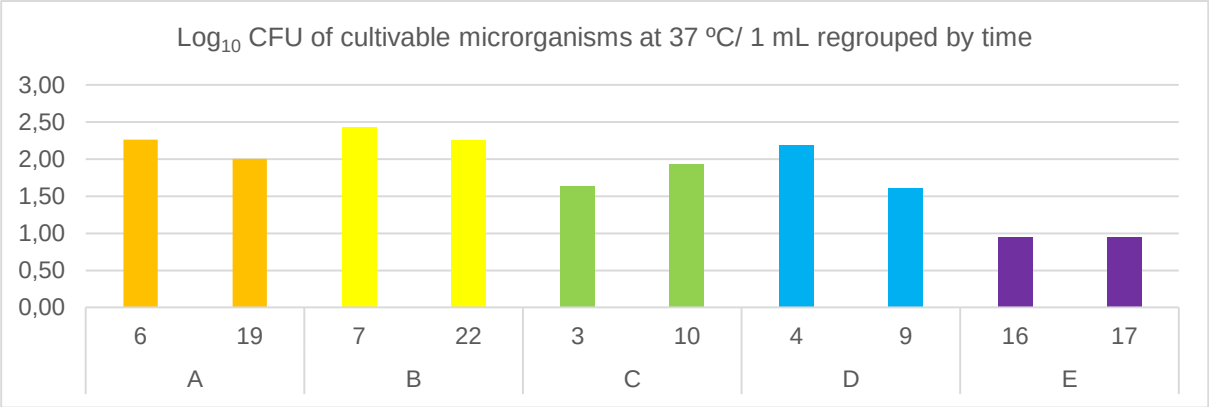


Figure 2. 15 - Comparison of results obtained from twice sampled waters coming from five dams, for cultivable microorganisms at 37 ºC (CFU log_{10} values by 1 mL)

The values for cultivable microorganisms at 37 ºC decrease in the second sampling in the A group (samples 6 and 19), B (samples 7 and 22) and D group (samples 4 and 9), remaining similar in E Group (sample 16 and 17). The C group (samples 3 and 10) reveal an increase in both cultivable microorganisms. The E Group had the same value for cultivable microorganisms at 37 ºC (an increase for cultivable microorganisms at 22 ºC), while samples B had the same value for the cultivable microorganisms at 22 ºC. The A, C, and D Groups had an increase in the second sampling for cultivable microorganisms at 22 ºC.

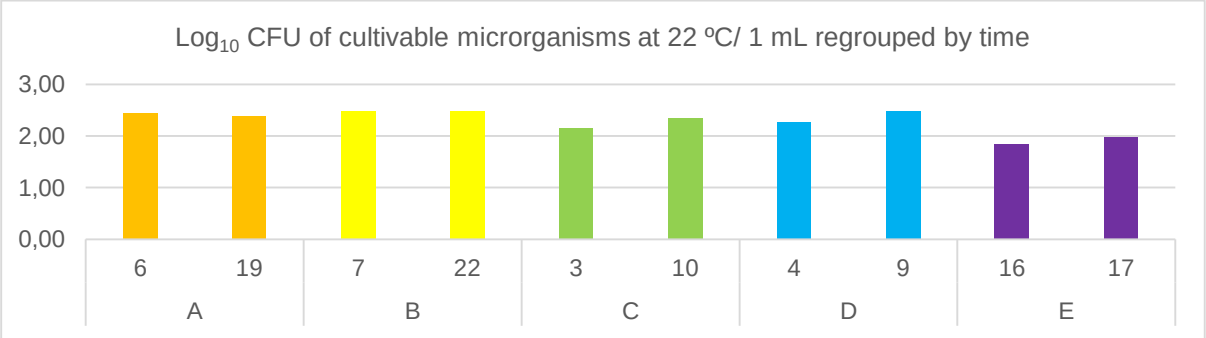


Figure 2. 16 - Comparison of twice sampled waters from five dams for cultivable microorganisms at 22 ºC (CFU log_{10} values by 1 mL)

For *E. coli* and coliforms, there was a tendency for a reduction in the value obtain for all the seconds time sampling in the same dam concerning A, B and D groups. In the C group only a small variation (reduction) was observed, a similar value was obtained. The E group showed the same result in both enumerations (Figures 2.17 and 2.18)

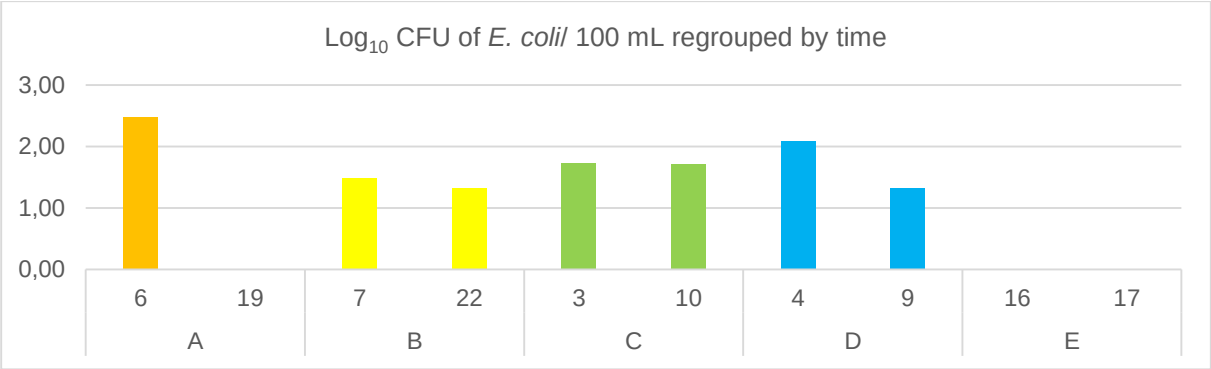


Figure 2. 17 - Comparison of twice sampled waters from five dams for *E. coli* (CFU log_{10} values by 100 mL)

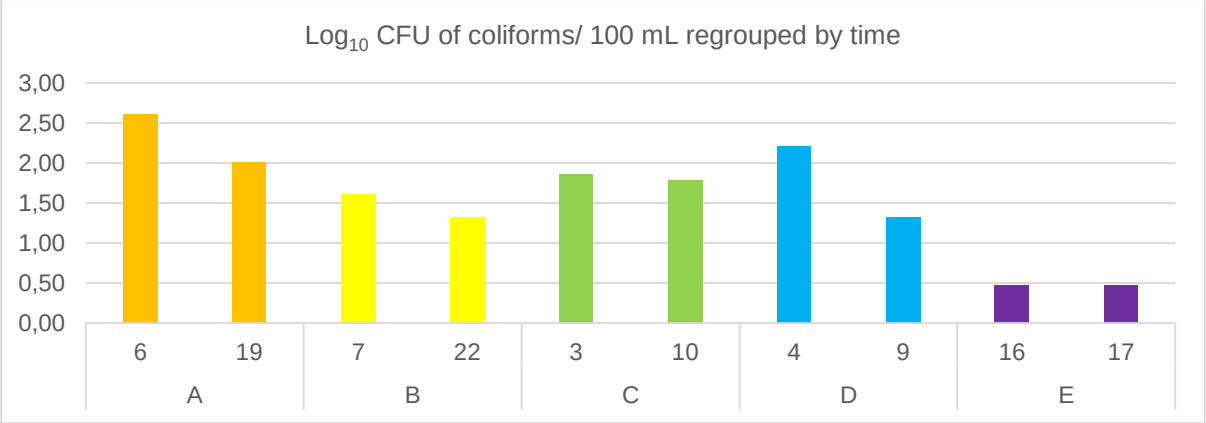


Figure 2. 18 - Comparison of twice sampled waters from five dams for coliforms (CFU log_{10} values by 100 mL)

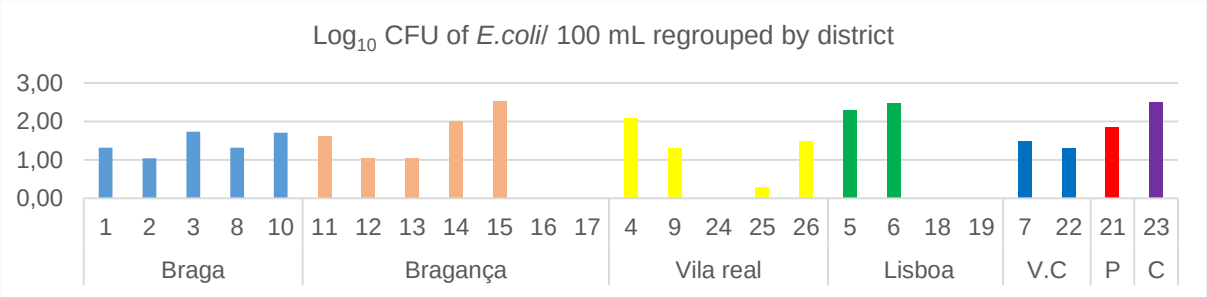
For *Enterococcus* spp. there was a decrease in the second samplings for the bacterial enumeration in the A and C groups (to zero). The D group showed a reduction, while B group had an increase. The E group this microorganism has been always absent (annex 8).

2.3.4.3. Comparison of samples coming from different geographic areas

A analyse was made to verify if there was any correspondence between the microorganisms and the geographic zones where samples had been collected.

The means values for the cultivable microorganisms at 37 ºC were similar among the Braga, Bragança and Vila Real groups and also between the Lisbon, Viana do Castelo, Portalegre, and Coimbra groups (annex 9). At 22 ºC the Braga group showed a mean of 2.21 ± 0.18 CFU/ 1 mL, in the Bragança group the mean value 2.15 ± 0.33 CFU/ 1 mL, Vila Real group had a value of 2.31 ± 0.28 CFU/ 1 mL, and Lisbon group 2.45 ± 0.05 CFU/ 1 mL. Viana do Castelo, Portalegre, and Coimbra groups showed the same value, 2.48 CFU/ 1 mL (annex 10).

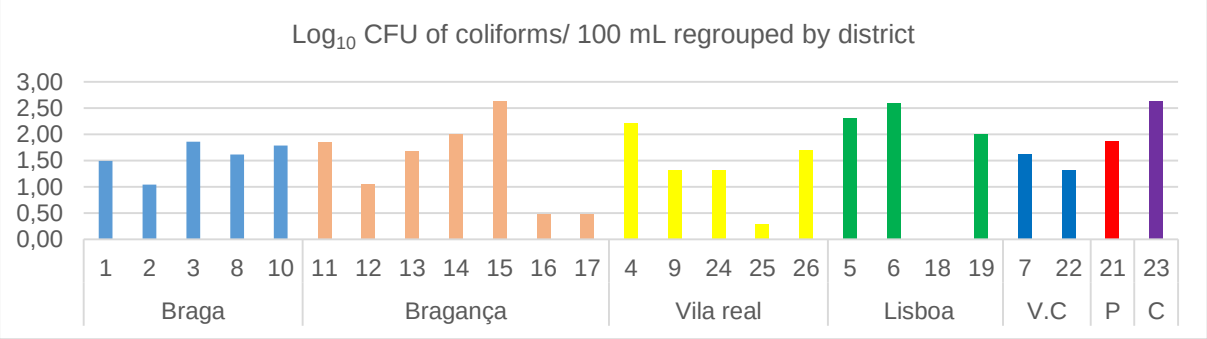
For *E. coli*, the highest values were obtained in the Coimbra district and Portalegre district with the single value 2.51 CFU/ 100 mL and 1.86 CFU/ 100 mL. The Braga district showed the value 1.43 ± 0.29 CFU/ 100 mL while Viana do Castelo district presented means of 1.41 ± 0.12 CFU/ 100 mL. The samples of Lisbon district and the Bragança district followed, with closer means among them (1.20 ± 1.38 CFU/ 100 mL and 1.17 ± 0.96 CFU/ 100 mL). The Vila Real district showed a value of 1.04 ± 0.87 CFU/ 100 mL (Figure 2.19).



Legend: V.C- Viana do Castelo; P- District of Portalegre; C - District of Coimbra

Figure 2. 19 - Comparison of results obtained for *E. coli* (CFU $\log_{10}$ values by 100 mL) using samples having different geographic areas of origin.

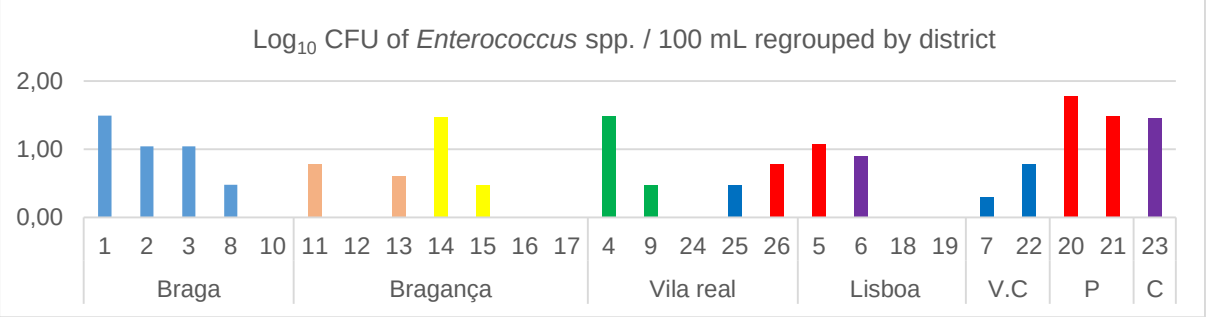
For coliforms, the highest value was also the Coimbra district (2.63 CFU/100 mL) and Portalegre district (1.86 CFU/100 mL). Viana do Castelo and Lisbon districts follows with the value 1.81 ± 0.21 CFU/100 mL and 1.64 ± 1.18 CFU/100 mL correspondingly. Braga district revealed a mean value of 1.56 ± 0.32 CFU/100 mL, Bragança group 1.45 ± 0.81 CFU/100 mL and Vila Real district showed the lowest value, 1.37 ± 0.70 CFU/100 mL (Figure 2.20).



Legend: V.C- Viana do Castelo; P- District of Portalegre; C - District of Coimbra

Figure 2. 20 - Comparison of samples having different geographic areas origins, for coliforms (CFU $\log_{10}$ values by 100 mL)

For *Enterococcus* spp., Coimbra district showed a single value (1.46 CFU/ 100 mL) that stand out from the others. Next comes the mean values from the Braga district (1.01 ± 0.58 CFU/ 100 mL) and Portalegre district (0.93 ± 0.21 CFU/ 100 mL). Vila Real district had the mean value 0.64 ± 0.55 CFU/ 100 mL, Viana do Castelo and Lisbon district, 0.54 ± 0.21 CFU/ 100 mL and 0.50 ± 0.58 CFU/ 100 mL respectively. Finally, the Bragança district had the lowest value, showing 0.48 ± 0.55 CFU/ 100 mL (Figure 2.21).



Legend: V.C- Viana do Castelo; P- District of Portalegre; C - District of Coimbra

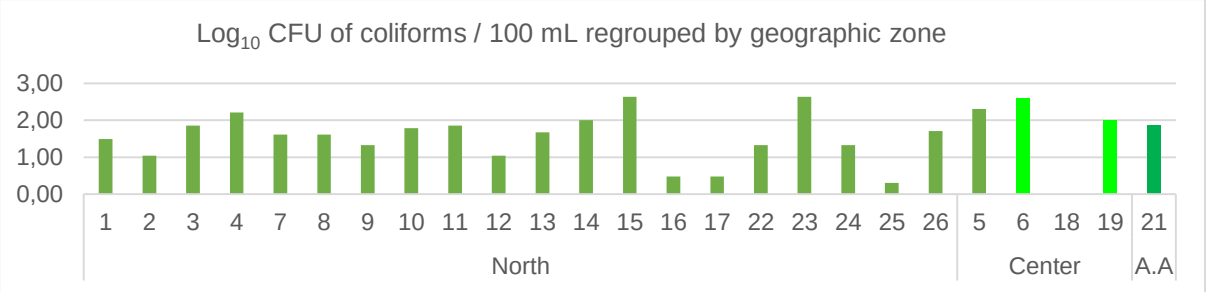
Figure 2. 21 - Comparison of samples coming from the different districts for *Enterococcus* spp. (CFU $\log_{10}$ values by 100 mL)

2.3.4.4. Comparison of samples collected in different administrative zones (NUTs 1)

The great majority of samples came from the “North” zone of the country, 20 samples, while only four samples were from the “Center. The “Alto Alentejo” group had one single value to *E. coli* and coliforms (1.86 CFU/ 100 mL), representing the highest value.

The group “North” presented the lowest mean value (1.81± 0.43 CFU/ 100 mL) for cultivable microorganisms at 37 ºC, while the “Alto Alentejo” group showed the highest value (2.48 ± 0.00 CFU/ 1 mL). The same tendency was observed for the values obtained for cultivable microorganisms at 22 ºC (in the “North” group the value was 2.25 ± 0.27 CFU/ 1 mL and in the “Alto Alentejo” group, 2.48 ± 0.00 CFU/ 1 mL) (annex 13 and 14).

The mean value for coliforms in the “North” and “Center” zone is higher when comparing to the *E. coli* mean (annex 11). The mean value is superior in the “Center” zone for coliforms (Figure 2.22).



Legend: A.A – « Alto Alentejo » zone

Figure 2. 22 - Comparison between samples from different zones for coliforms (CFU $\log_{10}$ values by 100 mL)

For *Enterococcus* spp., the mean value from the “Alto Alentejo” group was the highest (1.64 ± 0.21 CFU/ 100 mL) and the group “Center” the lowest (0.50 ± 0.58 CFU/ 100 mL) (annex 12).

Only cultivable microorganisms at 37 ºC showed a correlation with the zone ($p<0.05$).

2.3.4.5. Comparison of samples 16 and 17

Sample 16 and 17 were taken from the same dam for controlling of the quality of microbiological enumerations. The values obtain for *E. coli*, coliforms, *Enterococcus* spp., and cultivable microorganism at 37 ºC were equal. Only cultivable microorganisms at 22 ºC differ, but for very little (Figure 2.23 represents the relation).

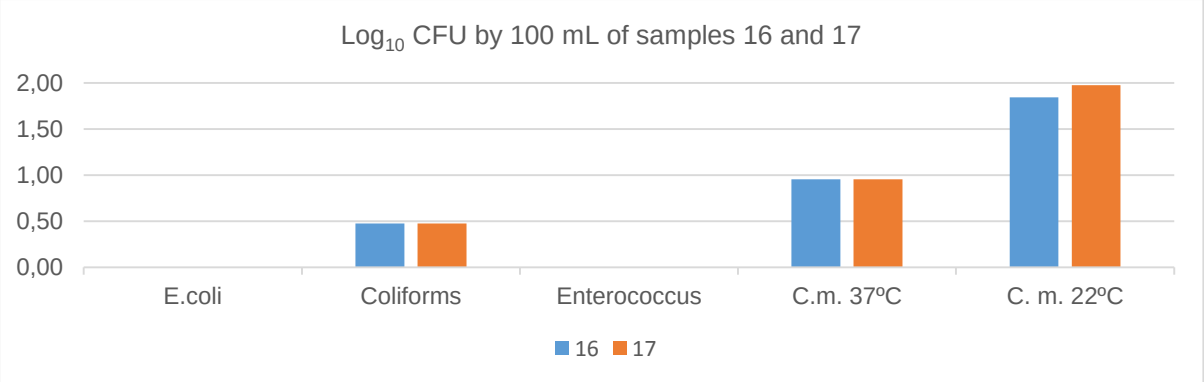


Figure 2. 23 - Comparison between samples 16 and 17 (CFU $\log_{10}$ values by 100 ml for *E.coli*, coliforms and *Enterococcus* spp.; CFU $\log_{10}$ values by 1 mL for cultivable microorganisms at 37 ºC and at 22 ºC)

2.3.5. Cyanobacteria and microcystin producers

It was observe first the presence of phytoplankton (Figure 2.24): Algae was found in 21 samples (80.77%) and cyanobacteria in 18 samples (69.23%). From this last group, 12 samples correspond to the evidence of the cyanobacteria genera usually associated with the competence to produce microcystins (46.15%).

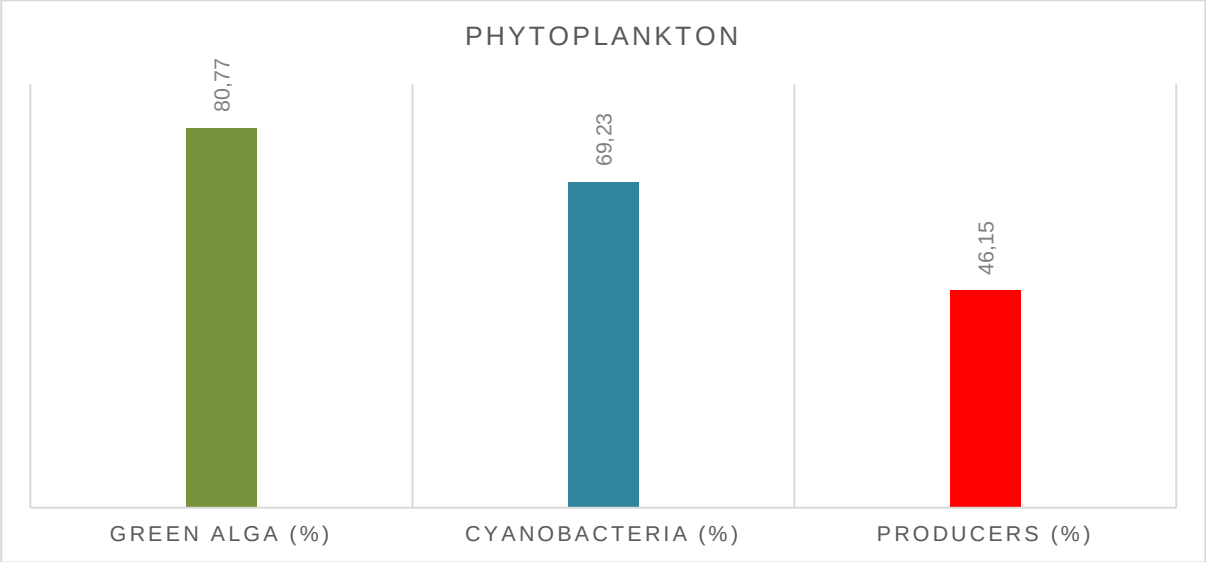


Figure 2. 24 - Frequency of Algae, cyanobacteria and microcystin producers (%) in the 26 water samples

The relative frequency of the chosen groups (microcystin producers) shows that *Microcystis* spp. was the main cyanobacteria present, in twelve samples (66.67%). The genus *Swonella* had the frequency 5.6%, it were present in one sample (Figure 2.25) (Table 2.6).

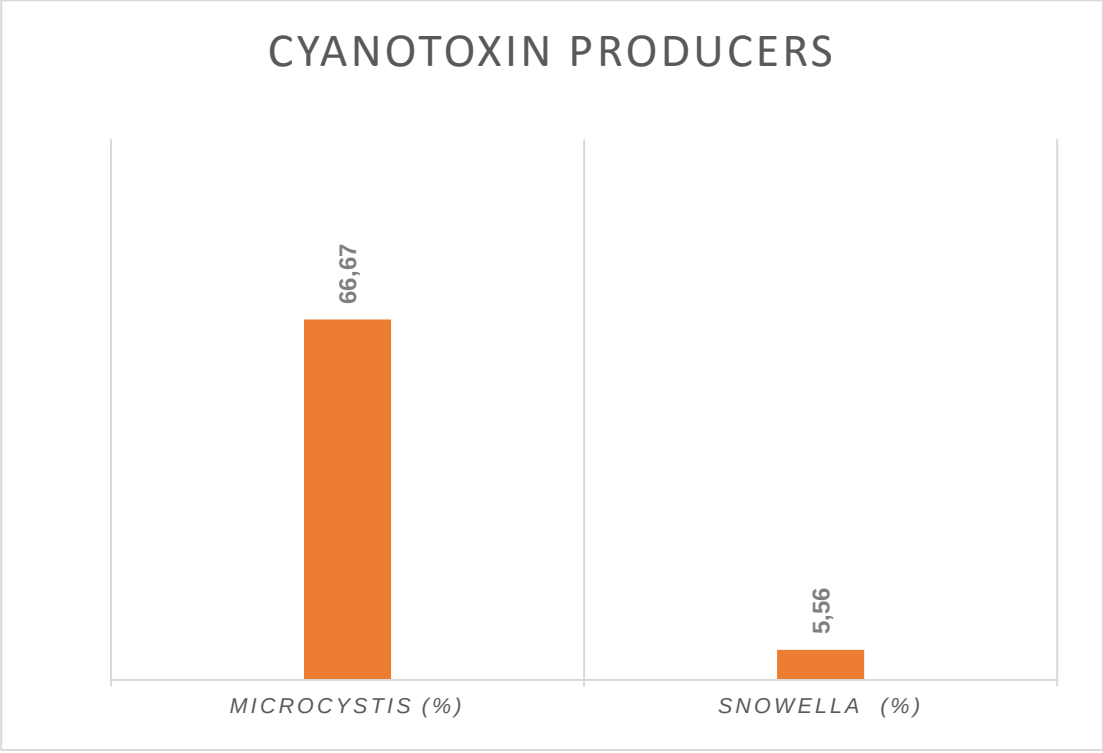


Figure 2. 25 - Relative frequency of the different genera of microcystin producers in the 18 samples

Table 2. 6 - Distribution by sample of the detected phytoplankton (samples 12,13, 14, 16, 17, are not represented, none phytoplankton was detect).

Sample	**Phytoplankton**			
	Algae	Cyanobacteria	*Microcystis*	*Snowella*
1	P	P	P	N
2	P	P	N	N
3	P	P	P	N
4	P	P	P	N
5	P	P	P	N
6	P	P	P	N
7	P	N	N	N
8	P	P	P	N
9	P	P	P	N
10	P	N	N	N
11	P	P	P	N
15	P	N	N	N
18	P	P	N	N
19	P	P	N	N
20	P	P	P	N
21	P	P	P	P
22	P	P	N	N
23	P	P	N	N
24	P	P	P	N
25	P	P	N	N
26	P	P	P	N

Legend: P = positive identification, N = negative identification

Examples of the groups refer are show forward:

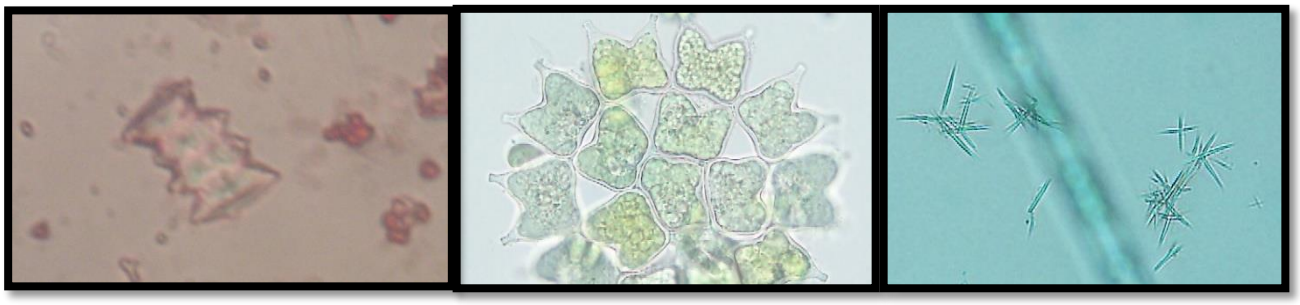


Figure 2. 26 - Examples of genera of algae detected – *Scenedesmus* spp., *Pediastrum* spp*., Ankistrodesmus spp.,* respectively (original 400x).

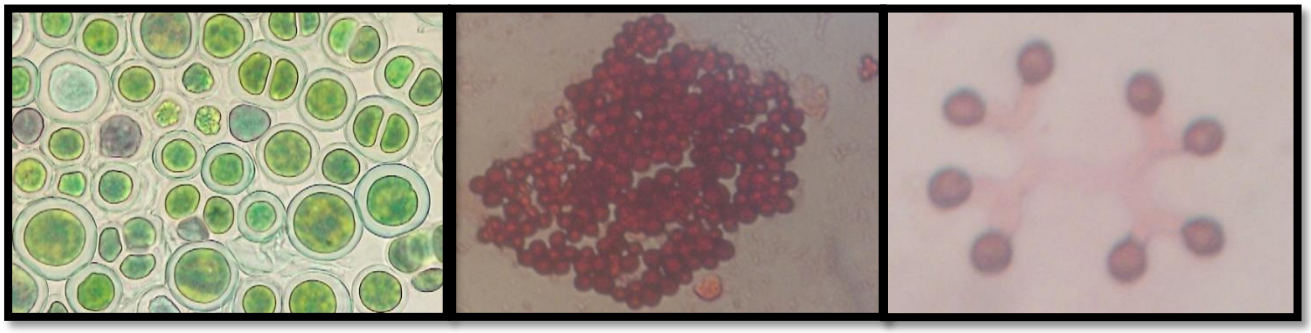


Figure 2. 27 - Examples of genera of cyanobacteria detected –*Gloeocapsa* spp., *Microcystis spp.*, *Snowella* spp., respectively (original 400x).

2.3.6. Bacteriophages

Bacteriophages of *Shigella sonnei* were not found in any sample, even after several tries. This kind of enteric bacteriophages were absent in all samples.

2.3.7. Classification of water quality

Concerning typification of water quality according legal bases and using *E.coli* as criteria for fecal contamination indicator, 13 samples had quality A1, 12 quality A2 and none had quality A3, when (Table 2.7).

Table 2. 7 - Classification of dams´ water quality using *E.coli* and its relative frequency

Quality	Number of samples	Frequency (%)
A1	13	50.00
A2	12	46.15
A3	0	0

When coliforms are used as criteria to classify the waters, 14 samples may considered as quality A1, 11 quality A2 and none had quality A3. Like with *E.coli,* coliforms are a group of bacteria that is used as fecal contamination indicator (Table 2.8).

Table 2. 8 - Classification of dams´ water quality using coliforms and its relative frequency

Quality	Number of Samples	Frequency (%)
A1	14	53.85
A2	11	42.31
A3	0	0

When *Enterococcus* spp. were used as criteria for fecal contamination indicator, 20 samples showed contaminations compatible with the A1 ranking, 6 quality A2 and none had quality A3 (Table 2.9).

Table 2. 9 - Classification of dams´ water quality using *Enterococcus* spp. and its relative frequency

Quality	Nº of Samples	Frequency (%)
A1	20	76.92
A2	6	23.08
A3	0	0

When using the assemble of the indicators to classify the water the prevailing classification is the one that detects the lowest quality. 10 samples showed contaminations compatible with the A1 ranking, 16 quality A2 and none had quality A3 (Table 2.10).

Table 2. 10 - Classification of dams´ water quality using all the indicators and its relative frequency

Quality	Nº of Samples	Frequency (%)
A1	10	38.46
A2	16	61.54
A3	0	0

Other microbial determinations, allowing obtaining real and relevant information to the water quality determination (cyanobacteria and bacteriophage of enteric bacteria), were not considered to classify the water safety levels, due to the lack of official criteria.

2.4. Discussion

A brief discussion can be made concerning the overall water quality, taking in consideration the global results obtained in this work; it was a main topic along this work. The general quality of Portuguese dams´ waters, attending to the results obtain on the present study, is quite satisfactory.

An aquatic biome is generally defined taken in consideration the physical and chemical characteristics, being freshwater when the concentration of salt is under 1% (rivers, lakes). The microbiota found in this place suffers an ecological pressure due to the temperature, pH, flow rate, light intensity and nutrients [193]. The waters of dams are colonized by typical native microbiotas. They are also quite variable with the geographic location of the artificial lake and the longitudinal and vertical zonation of each specific water column [193 – 195]. A thermal stratification may occur in some seasons (especially in summer) [197].

The surface water absorbs solar energy for a long period, forming distinct water zones: the epilimnion (surface layer), thermocline - point of the metalimnion (middle transition layer) in which the temperatures change quickly with the depth - and hypolimnion (bottom layer). An important factor to the natural microbiota and their metabolic reactions are the changes in temperature. Some strains from colder water (from the river above) can substitute them, if the temperature in the dam are not adequate [197].

The warmer water is present in the epilimnion in a thermal stratified water system, and the more colder and dense in the hypolimnion (without mixing). Thus, deep waters do not receive oxygen and remain in hypoxic conditions, separated from the surface by the interface zone. The nutrients and contaminants in the soils can reach the upper zone, if a mixing occurs (heavy rainfall or discharges). This excess of nutrients is a promoter of the bacterial growth, reducing the overall water quality, and the ecosystem equilibrium (e.g. algal blooms, odors and subaquatic animal) - stratification is clearly a factor with a strong impact in waters [194]**.**

The stratification of the water had an influence in the microbiological indicators in the water column (*E. coli* and fecal coliforms) in a study performed from April to September, in an analysed lake zone (transitional zone of the dam-lake) (the Kardzhali reservoir). In the bottom layers there were higher numbers of both microorganisms, because they are connect to colloid sized small particles that have the nutrients. The temperature, itself, had an effect, because the survival of fecal coliforms (FC) is higher at low water temperatures; higher temperatures in an environment with high concentrations of dissolved oxygen (as the euphotic water layer) induce an inhibitory effect [198].

Besides the role of natural indigenous microbiota of the waters of dams and their promotors' ecological factors, there are other sporadic exogenous microbial contaminations, including some potential pathogenic agents. Some exogenous contaminations are from human responsibility. In the Kardzhali reservoir there was evidence of an increase of the number of *E. coli*, fecal *Streptococci* and *C. perfringens* in July and August, in sintony with the higher tourist activity in this season [198] **.** The pollution of aquatic systems are increasing with the growth of industrialization along the years (and the increase of population). Industrial waste materials, agricultural insecticides and surplus fertilizers may achieve the water resources and contaminate food resources, affecting humans [199]. This external contamination may represent potential risks for the human health.

Monitoring all the normal or pathogenic microorganisms that may be present in the water of dams is not a practicable and efficient way to access water safely; there are several variables, from exogenous contaminations, human contaminations and even ecological factors [201]. The impact of pathogens are, in a succinct way, due to the contamination and intensity of the sources, but also due to the persistence and transport of the pathogen in the site and resistance to the treatments apply [200].

To point and correlate all these with the results obtained in studies and scientific works is a demanding task. The objective of this work was to help in the characterization of dams' water and possible explanations for the results.

The most practicable microbial control able to achieve accurate information concerning the presence of microbial pathogens in those waters is based on the use of indicators, allowing access to plausible predictions [64]. The enumeration of indicators to estimate the amount of contamination in water is preferred over directed pathogens surviving in similar ecological conditions (physical, chemical or biotic interactions), as they are considered to be normal (non-pathogenic) intestinal inhabitants, in high numbers and are technically easy to detect and enumerate [66].

Indicator microorganisms reveal a hypothetic presence of other pathogen agents, cohabitants of the same original ecosystem, allowing a cheaper and easier assessment of both, fecal or environmental, disease promoter's relevant microorganisms. The economic factor is the biggest limitation to conduct systematic analyses of water, in regions in development. The less expensive analytic procedure, permitting accurate results, is always the most desired methodology. This is a criterion that is also taken in consideration when the target is the microbes used as indicators, in waters [54].

There are cases in which this correlation is not found or unclear results are obtain, because the absence of the indicator does not mean, for sure, the absence of the pathogen. Evidences between a certain indicator and the source of the pathogen, or it potential to cause a disease, are another limitations [202]. Different indicators diverge in the fundamental ability to predict human risks. Therefore, choosing one single "perfect indicator" is unreliable. All the bacteria used and bacteriophage were intended to give a broad spectre of the water quality and better characterize it. The individual characteristics are further appointed.

It must be emphasized that the cultivable microorganisms are important indicators of general level of contamination (including organic), a parameter that is appropriate to evaluate the water quality. Exogenous contaminations of waters are relevant, and total cultivable microorganisms represent the parameter that expresses the general microbial burden, assuming the limitations of the technique. Thus, the vast majority of the microorganism introduced in the water is detected by the enumerations procedures, which could not be achieved if some single specific microbiological indicators were used. This parameter is relevant to assess the application of the most appropriate treatment to the waters in an away to obtain a safe final product.

Enterococcus spp. and *E. coli* are the most frequent indicators for fecal contaminations of waters. Total coliforms indicate environmental contaminations and cyanobacteria provide information on the level of eutrophication, also setting specific health risks. Finally, bacteriophages of human enteric bacteria are indicators of fecal contamination or the potential presence of human enteric viruses. All the

procedures for detections or enumerations of microorganisms in water followed simple procedures ensuring repeatability and reliability of the methodologies.

Sampling methodologies were accomplished aseptically using sterile glass bottles and transported under conditions, allowing the stability of the samples. The volume transported sample (about 900 mL in a bottle of 1000 mL) was sufficient to allow an adequate homogenization. Water volume losses during packaging and transportation were not detected. Direct individual sampling was carried out, whenever samples collected professionally were not available, in order to maintain the schedule of the analytical program. However, those sampling procedures were collected at the edge of the coastal (limnic zone) of the water column of the dam and not at a point in the limnetic zone.

Artificial ponds are lentic systems, but are expose to rainfall events and are generally connected to a river, which gives them similar characteristics to lotic systems (rivers, streams). The dam water may accumulate exogenous nutrients and sediments during the rainfall. The sedimentation is higher near the shoreline (margin) and agriculture practices, mining and industrial practices near the water dam may occur further nutrients and sediment accumulation [203]. These nutrients near the margin promotes the microbiota growth, and so, samples collected at the water's edge, obviously do not have the exact same level of general microbial burden. It may have microbial contaminations coming from the benthic deposits, effluent effluxes and from the coastal aquatic flora and fauna, providing higher levels of microbial burden.

Because the microbiota composition is always changing, the time recommended between the sampling and laboratorial processing (transit time) should not surpass 6 hours. It has been stated by the competent official authorities, or a maximum of 24 hours in refrigeration (NF EN ISO 9308-1 and NF EN ISO 7899-2). This lapse of time was not always possible to respect, due to logistic and operative technical reasons, being prolonged for some days in some samples. The prolonged time of refrigeration may have affected the results, being plausible to admit that some microorganisms may have been inactivated and other may have been favoured.

The samples were put at room temperature and under the incidence of natural light, before the procedure. These higher temperatures were intended to reduce eventual negative effects possible attributed to colder temperatures. The procedure was initiated at the time of samples arrival, concerning to cyanobacteria search, water was transferred to the final correct bottles, as a way to restrain this microorganism from adverse grow conditions.

The different culture media were extemporaneously prepared in local laboratorial conditions following the manufacturer instructions, to minimise the number of variables influencing the results. The media and reagent need for the workload week cycle, were prepared each week or for the subsequent one (stored in refrigeration conditions after sterilization), to ensure that the culture media remain stable and at the adequate conditions.

The fact that sampling procedures were executed during autumn and winter, for a temporal period of four months, may also had some influence on the results. The basin of the dam may be reached by inflows of rainwater, bringing more debris, affecting water transparency and its biochemical composition. Dams located in a basin having industrialized zones or large urban agglomerates also may be prejudiced by the microbial contamination of its waters, as already referred, since they can be the

source of polluted inflows; especially if the effluents were not sufficiently treated or decontaminated.

It is important to comment the influence of the season and the relationship with rainwater. The torrential rain flow may contain some pathogens after the storm and the use of bacteria indicator, in this case, is still unclear. It is generally recognized that microbial values are higher after storms, and some epidemiological studies reveal that when the humans are expose to contaminated marine water, it had some adverse health effects [204].

Not only the sampling procedure and the intrinsically differences (season, geographic zone, sampling place) but also the multiples detections, isolation and identification methods have been implemented aiming to obtain accurate results.

Membrane filtrations techniques are the main procedures for monitoring microbial characteristics of water, being recommended in literature and guides [205], especially when the number of microbes is few, a concentration is need to detected them. The Membrane Filtration (MF) Techniques were introduced in 1950. U.S. EPA accepted this technique in the 11th edition of Standard Methods for the Examination of Water and Wastewater. The Microbiological Methods for Monitoring the Environment had stated, in 1978, that the MF Technique is ideal for water testing, because it permits analysis of larger samples in less time. It is an alternative to the “Most Probable Number” (MPN) procedure, although with higher sensitivity. Has also the possibility of isolating discrete colonies of bacteria while the MPN procedure only indicates their presence or absence (indicated by turbidity and gas in test tubes) [206]. Selected dilutions were used intending to obtain microorganisms growth within the counting limits (30 - 300 CFU / mL). Total cultivable microorganisms inoculums determinations never needed dilutions, as the maximum value 300 were considered the limit for calculations.

Filtration methods are indispensable when the targeted microbes are in a very low concentration in a liquid matrix and higher sensitivity is demand. It is a simple and low cost procedure, but, like all methods, they have some intrinsic limitations. It is a laborious method when several samples have to be processed; solids in suspension in water (sediments) can prematurely clog the filter membranes, affecting microbial adhesion, and later, inhibiting or interfering with the bacterial growth or the enumeration [206] and finally, culture media do not have always the necessary selectivity, allowing the grow of others non targeted bacterial species. In the case of sample 20, for example, it was not possible to count all coliforms, since “non-coliform” bacteria were widely predominant.

The membrane filtration revealed to be useful and practicable in this work, even attending to the limitations previously referred. Problems were registered only with one sample, processed for coliforms and *E. coli*, since it was unable to allow the accountability. High levels of cohabitant microbiota burden, in this specific case, putting problems to the discrimination of the target agents (sample 20).

These techniques are generally in practice for water analyses and proved to be an efficient methodology. Some complementary chemical tests may assist in mitigating some of the limitations attributed to conventional microbial methodologies. They detect directly chemical hazards or chemical indicators. They were not utilized in the present work. Some chemical treatments applied directly to waters, like disinfectants (biocides quantification), expecting to help its total microbiota reduction, may help to explain an eventual lower level of total microorganisms in those waters.

Levels of chlorophyll or dissolved oxygen are other examples of chemical parameters relevant for the indirect microbial estimation. Some chemical tests, like those previously referred, are supplemental method allowing to obtain evidences of possible factors that may have an impact on the results, but this work had only a focus on the microbiological parameters to characterize the water of dams. Chemical tests require specific laboratory premises and expensive equipment's that was not available at the time that this work was elaborated. The microbiological parameters are not believed to be the only parameter needed to classify the safety status of waters if dams. Other parameters may be applied to help in a most exhaustive monitoring; they are believed to be correlated with problems in water quality or fecal contaminations.

The plate count method was applied for enumeration of cultivable microorganisms at 22 ºC and 37 ºC. With the pour plate technique was possible to determine the number of microorganisms find in a sample. It is a quite simple procedure, allowing an idea of the general microbial burden present in water; to perform it, very accurate specific skills are not required. The major challenges are the correct or uniform distribution of the inoculums having the bacteria by the plate area, incorporated in an agarose that must not solidify neither inactivate microbes (a critical temperature near 48 ºC). The universal culture medium molten at the critical temperature, in a very succinct explanation, it is added to the Petri dish were 1 ml of sample volumes (and decimal dilutions) has been previously dispensed. The dishes were then covered with its lids, mixed gentle for inoculums incorporation and wait till solidification of the gelose. The number of colonies is counted and is referred as colony forming units (CFU).

The incorporation of the inoculums for cultivable microorganism, concerning the present work, was carefully achieved. The temperature of the culture medium was not high enough to inactivate the agents allowing an adequate distribution of them: conflicts in the enumerations due to overlapping bacterial colonies, were rare. Aseptic conditions were seriously taken in consideration (like in all protocols); fungal contaminations could affect the results, since colonies dimensions sometimes overlap all the other microorganisms, not allowing its enumeration. The test itself does not specify the identity of the organisms that are detected.

Cultivable microorganisms are bacteria that use organic compounds as a source of energy and carbon, comprising bacteria that grown on the chosen medium under the specified conditions [207]. They are a major and widely used means to assess the microbiological quality of water [208], e.g. bacteria accounts at 37 ºC can provide an early indication of exogenous pollution.

It is an easy and quick procedure, whose disadvantages may also be pointed. The first difficulty is to monitor the temperature of culture media (Yeast Extract Agar): if it is too high may cause heat shock and bacteria become inactivated ("non cultivable"); second limitation concerns to the fact that only planktonic cells are transferred; third problem is the aggregation of different number of bacterial cells that remain physically linked even in the subsequent growth (reason why the results are expressed in CFU/ ml); fourth question is the condition of incubation (temperature, time and aeration), allowing mesophilic aerobic bacteria to multiply but denying this possibility to anaerobic agents or those having other spectrum of thermophylia; the nutritional composition of the agaroses, probably microbes demanding accessory nutritional requirements do not have the possibility to multiply; and finally, biotic

interactions and competition among bacteria growing simultaneously in the same matrix may enhance error factors.

All these aspects are attainable, allowing to state that only a part of the total microorganisms present in water samples may have the chance to express its presence within this analytical procedure. The location of the sampling point and season are accessory factors that may have an influence in the heterogeneity of the results. The total microorganisms recovered include both, indigenous microbiota and microorganisms coming from contaminating sources [209].

The evaluation of total cultivable microorganisms was made looking for limit values and assuming that a significant difference is due to temporary and not predictable factors, among them sampling location (the points are previously selected in dams) and the analytical protocol is standardized (temperature and culture media).

The parametric references do not take in consideration the genus of the microorganisms that have been found. This is not the aim of the analytic procedure, because what matter in this case is the general quantity, assuming its correlations with organic disturbances occurring in water.

To minimize perturbations or confusions concerning the results and, as require for the protocol, the samples were always processed in duplicate, being used the mean of their values in order to represent the result obtained. Duplicated samples help to confirm the repeatability of the analytical procedure, since both values have not been much different.

Correlation obtained between the two cultures of same sample for cultivable microorganisms was performed; a positive correlation was found between them ($rho=0.62$, $p<0.05$).This was expected because the microorganisms quantification were almost equal in both determinations with the same sample (resist and growth at the same ecological conditions). All values obtained for cultivable microorganisms at 22 ºC were superior than at 37 ºC , as was expected, since microorganisms came from natural environments. Only in sample 12 there was a higher value of cultivable microorganisms at 37 ºC, this is not frequent in waters, and may represent a laboratorial error.

The points where sampling collection took place could have affected the results as previously referred, so the mean values of the results of total cultivable microorganisms were compared to verify if these collecting points had systematically different values. Only cultivable microorganisms at 37 ºC showed a correlation with the sampling point ($p<0.05$), using statistic analyse, which means that the sampling place could had impacts in the results. Higher levels of cultivable microorganisms were detected in samples collected directly in the littoral margin of dams.

These events may be due to the proximity of the benthic environment that have higher concentrations of microbiota coming from the soil, aquatic fauna and flora. Nevertheless, this can also be due to the environmental contaminations, because cultivable microorganisms at 37 ºC are more associated with exogenous pollutions [210], like fecal contaminations.

The climatic conditions can also influence the resistance and survival of the microorganisms; for cultivable microorganisms at 22 ºC the values can be superior in the winter season due to inflows of rain water drained through the basin, dragging higher concentrations of nutrients and affecting water turbidity. Natural UV radiation is not able to penetrate in water, in this condition, allowing higher rates of microbial survival [211]. An increase in the mean values of total cultivable microorganism was registered

in the winter season. The number of cultivable microorganisms at 22 ºC showed a positive seasonal correlation ($p<0.05$). Comparing the results that were obtained with different time of sampling from the same dam, the majority of the seconds' collections revealed an inferior value for cultivable microorganisms at 22 ºC. This tendency, contrary to expected, does not seem to have any special meaning. This achievement may be justified to the fact that the winter of 2015 had been a quite dry season, without significant raining volumes [212].

The means values for cultivable microorganisms at 37 ºC were similar between the group of samples collected in Braga, Bragança and Vila Real districts, and between the groups collected in Lisbon, Viana do Castelo, Portalegre and Coimbra districts.

The groups of samples coming from Braga, Bragança and Vila Real districts were closely relate places and presented mean values inferior to the samples coming from Lisbon and Portalegre, to both cultivable microorganisms. This was expected because these are samples from the direct sampling.

Cultivable microorganisms at 22 ºC do not seem to have so obvious differences between districts; but the values were higher in those districts from south.

They were regrouped in larger geographic zones of distribution ("NUT 1) and were statistically analysed, because each district distribution concerns to groups having a small number of samples.

The sample's group of "North" presented the lowest mean value for cultivable microorganisms at 37 ºC (1.81 ± 0.43 CFU/ mL), while the single sample of "Alto Alentejo" showed the highest value (2.48 ± 0.00 CFU/ mL). The samples collected in "North" zone were professionally collected, as previously referred.

The same tendency was observed with the values that were obtained for cultivable microorganisms at 22 ºC ("North" group having 2.25 ± 0.27 CFU/ mL and "Center" group showing 2.48 ± 0.00 CFU/ mL). Samples coming from "Lisbon and Tagus Valley" presented values closer to those observed in the samples coming from the "Alto Alentejo" zone for both cultivable microorganisms.

The number of cultivable microorganisms at 37 ºC showed a positive correlation with the zone ($p<0.05$), supporting the idea of some factor influencing the general microbial burden in those waters.

The factor itself cannot be elucidated, but is possible to comment that the samples from the Center (Lisbon, from margin sampling) had higher values. These higher values can be explain by the microbiota present in the soils near the margin or waters runoffs (higher population density).

Looking exclusively to the mean values by zone or to the single value of a specific analyse can be insufficient. Water quality evaluation based on significant changes in their total microbial number has a clear utility. To do it, systematic microbial monitoring is necessary, trying to detect earlier disturbances that may occur due to multiple reasons, if the same method is used. An inadequate water treatment may be detected through to this systematic control, when the number of cultivable microorganisms becomes suddenly higher than those usually observed.

The relatively reduced number of samples is a relevant limitation to accomplish a temporal variation in this kind of investigation, since only few samples came from the same dam. The results that were obtained may give a general idea of the usual number of microorganisms that may be present in the waters of dams in Portugal, even without a longitudinal sampling plan, allowing also establish correlations with the values that were obtained for other microbial indicators.

Predictions about the water quality or if the treatments are efficient are essential and the presence of extent fecal contaminations is another important factor to assess the water quality and the potential of risk human infection. The presence of coliform bacteria, although it is not a proof of fecal contamination, may indicate a failure in treatment, storage, or distribution. Examination of water samples for the presence of *Escherichia coli*, which normally inhabits the bowel of man and other warm-blooded animals, is an indication of such contamination. Not only humans are affected, all the macrobiotic depending of the dams´ water are exposed (e.g. fish are exposed to several bacteria that occur in surface waters [213]). Examination of such indicators can not only protected human health but also guaranty the survival of the animals that use dams´ waters as a habitat or resource.

The procedure to identify the total coliforms was based in the characteristic yellow color of the colonies on Tergitol Agar in the sequence of Lactose fermentation and consequent acidification of the matrix and the confirmative oxidase text (negative). A number of 10 yellow colonies, at least, were always subject to isolation to ensure a significant and representative number. No metal loop was used because it may give false-positive results, and, as an additional control measure, the oxidase reagent was extemporaneously prepared in the same week as the test performance and stored for a maximum of two weeks prior to use. Only these two characteristics were used for the identification, even with these possible factors perturbing, since they are considered enough for this functional group of microorganisms. No distinction at the level of species or genus was performed, being, this procedure, sufficient to confirm their presence.

E. coli identification and account were achieved using coliforms colonies (having a yellow color on Tergitol Agar and oxidase negatives), then, at least 10 colonies were selected and inoculated in Brilliant Green Broth and peptone water. Finally, the indole test was performed after an “overnight” incubation at 44.5 ºC. The formation of a bright pink ring on the surface of the broth culture was considered a positive result. If other, different color appeared (e.g. yellow), it meant the presence of other microorganisms; the color absence is explain by the fact that the microorganism does not have tryptophan – deaminase, enzyme need to indole release. Other water native microbes, like *Aeromonas hydrophila*, *Aeromonas punctata*, *Plesiomonas shigelloides* and *Vibrio* spp., are equally indole producers, but they are inapt to growth at 44.5 ºC with 2% of bile salts.

Total coliforms represent both fecal and environmental contaminations in waters (e.g. bacteria colonization of plants or animals). When the results are correlate with the values obtained for *E. coli* it can better differentiate between the two types of contamination. In a study regarding waters of mountains areas, it was demonstrated that *E. coli* and *Enterococci* are ubiquitous in feces from human and animal (livestock or wildlife), being reliable indicators for water resources [214]. This study focused in the animal life that may contaminate a water course and gives insight about the stability of *E. coli* as an animal fecal indicator. *Clostridium perfringens* occurred only in human, livestock and carnivorous source groups in relevant average concentrations (but not in herbivorous wildlife sources).

High values of total coliforms or *E. coli* might indicate the possible presence of a microbial hazard in the water, threatening life and after surpassing a given limit. Action plans must be established, so that the water became fit for use. It means samples having predominantly *E. coli*, when the values of coliforms

and *E. coli* are the same. It may be the result of more environmental contaminations (phytophilic bacteria) or others “not identified” coliforms, if only the value of coliforms is higher (it is never lower).

Samples number 16, 17, 19 and 24 only revealed coliforms. They are not always related to fecal contamination, so it is not surprising if they are identified in the absence of others fecal indicators (*Enterococcus* spp. was also absent in these samples).

Official limits used for coliforms are, obviously, superior to the value of *E. coli*, regardless its significance in terms of health risk. A strong positive correlation was found between coliforms and *E. coli* (rho = 0.85, $p < 0.05$). That is generally expected, since the value of coliforms includes the *E. coli* values, as part of the functional group.

The samples collected directly in the dam margins showed a superior mean value for both, coliforms and *E. coli*. This may be attributable to higher fecal exogenous contaminations of the water itself (the sampling place did not show a statistical correlation for both group of microorganisms).

The mean values obtained in the autumn and winter season were superior in the first group refer to *E. coli* and coliforms probably due to the fact that the winter season of 2015 has not been very rainy (water became more transparent) [212], contrary to the autumn [215].

Analyses from a reservoir in Southern California, USA, over the period August 2001-July 2002, showed seasonal trends in bacteria concentrations (greater numbers and heterogeneity of total and fecal coliforms, especially during the summer) [216].

No statistical correlation was found in the present work, and this superior value may be due to a more mild temperature favouring both, coliforms and *E. coli*.

When sample partition was analysed using the district division, the highest value for coliforms was detected in single samples coming from dams located in Coimbra and Portalegre districts. The samples from Viana do Castelo and Lisbon also revealed high levels of coliforms contamination. Samples from Portalegre and Lisbon have high mean values probably due to its different sample procedures (direct sampling). The samples from Braga, Bragança and Vila Real presented the lowest values.

The highest values, for *E. coli*, were found in samples coming from Coimbra and Portalegre, like the results obtained for coliforms, because the main detected coliform was *E. coli*. Braga has here a higher mean value when comparing to coliforms, again because E. coli was the main microorganisms present, representing possible fecal contaminations, instead of an environmental contamination. Viana do Castelo and Lisbon have still high mean values of *E. coli*, representing too a potential health risk. Bragança and Vila Real had again the lowest values, represents the less contaminated water with *E. coli* and coliforms.

The highest values for coliforms and *E. coli*, simultaneously, were found in samples coming from the “Alto Alentejo” region: a single sample showed the same mean value for both determinations, in which all the detected coliforms were *E. coli*, confirming fecal contaminations. The lowest mean values for coliforms was observed in samples collected in “North” region, while for *E. coli* was the “Center” zone, meaning probably that samples from this zone were probably more expose to environmental contaminations (due to higher forest density), instead of potential fecal contaminators.

The filtration procedure was repeated with a new decimal dilution of the water, in the few cases in which the two dilutions were not enough to obtain an accurate result, attempting to solve the difficulty. The sample 20 was uncountable for both group of microorganisms even with supplementary dilutions.

The combination of these two indicators, using a unique protocol is a huge advantage and do not constitute an elevated cost. The enumerations were easily performed, being recommend to do at least two dilutions for each water sample. This may help in situations where the expected number does not correspond to values previously predicted.

Another fecal indicator group that has been used, for exogenous water contaminations, is *Enterococcus* spp.. Some species of the genus *Streptococcus* (namely *S. bovis* and *S. equinus*) may occasionally growth in the analytic conditions of this study, but they do not have the same eco-resistance pattern of *Enterococcus* spp. species. Its detection through the analytical procedure is improbable.

Ecological resistance of *Enterococcus* spp. is superior to the previous fecal indicators [74], supporting higher salt concentrations, more extreme temperatures or pH values. The meaning of its presence in water of dams needs a prudent interpretation. The microorganisms that are considered to be indicators should have the same eco-resistance pattern of the pathogenic microorganism that it is supposed they indicate. Theoretically they must not be present when the indicated pathogen disappeared in the same matrix and it, no longer, represents a health risk. On the other hand, a microbial indicator that resists in different ecological conditions is useful in more than one kind of water (e.g. drinking waters and recreational water samples).

The samples that have been collected directly, near the margins, had higher mean values of *Enterococcus* spp., but without statistical correlation. This result is similar to those obtained with the previous indicators and again, the explanation may lie in a more general contaminated water; as it is seen in the high values of total cultivable microorganisms in these waters samples.

Concerning to the levels of *Enterococcus* spp., a decrease in the second sampling was registered in samples coming from the districts Braga, Lisbon and Vila Real. Once again, lower rainy season [212] and higher water transparency may assist the justification of the findings. Viana do Castelo dam had an increase. From the samples being obtained from Bragança dam, *Enterococcus* spp., were always absent.

The winter season showed a superior mean value, but without statistic correlation. This was expectable since *Enterococcus* spp., can resist to more extreme conditions [180] [201]; it was a very dry season.

Concerning to samples distribution by district, the sample means obtained in Portalegre, stands out from the others and the single value from Coimbra. The high values in enumerations from these districts had already been registered with coliforms and *E. coli*.

Samples displaying the next higher values came from the districts Braga and Vila Real. Samples collected in the district Vila Real revealed a mean value of *Enterococcus* spp., superior to the district Viana do Castelo and district Lisbon, which is different from the tendency observed with *E. coli*. These events represent, probably, differences in the detections of the various indicators, but appears to be a correlation between the values from both indicators. Finally, the district Bragança showed the lowest value, like in coliforms and *E. coli*, these samples had lower values, in general.

The mean values for *Enterococcus* spp. show higher levels, considering regional distributions, in the samples coming from the “Alto Alentejo” and the “North”. Samples from the “Center” region showed the lowest. This tendency is similar to the results registered for *E. coli*. The figures are very satisfactory because they show a good strong positive correlation between the two indicators; instead of confusing the results, they seem to be interconnect.

Comparing the present results with other obtained in different regions of Earth, like those in the Alau Dam Maiduguri, Borno State, Nigeria, where microbiological parameters were registered: enumeration of cultivable microorganism at 30 ºC, coliform, *E. coli* count and *P. aeruginosa* [217]. The enumerations were made in five sampling point (point II was water from Lake Alau). Only the point V (treated water) reveal lower cultivable microorganism count (110 CFU/ ml) and all the others microbial groups were absent. Water taken directly from the lake had the higher count for all microbiological parameters, cultivable microorganism at 30 ºC were 430 CFU/ ml, coliforms 180 CFU/ ml, *E. coli* 110 CFU/ ml and *P. aeruginosa* 30 CFU/ ml.

Cultivable microorganism at 30 ºC was associated to irrigation, fishing and animal grazing activities as well as washing activities taking place on dam basin. Six samples, in our work, also reveal *E. coli* and coliforms values equal or superior to 100 CFU/ mL, similar to the results obtained in the previous study, having a probable origin in fecal contaminations.

In the part of the Eastern Cape Province, the Bufallo river and the associated dams (South Africa) are a source of water for needed irrigation, recreation and consumption. 3 dams and water from the river were microbiologicaly analysed for 12 months, between August 2010 to July 2011 [218].
The total count were high, ranging for total coliforms 6.6×10^{1}-3.8×10^{7} CFU/ 100 ml, fecal coliforms 3.0×10^{1}-3.0×10^{5} CFU/ 100 ml and *Enterococcus* spp. 3.4×10^{1}-5.3×10^{4} CFU/ 100 ml. The values were higher for all indicators, an uniform trend, especially in the lower reaches of the Bufallo river. This was consider associated with the more intense anthropogenic activities, and elevate concentration of the populations in that zone. The impact of the rural and urban population, without adequate monitoring and adequate sanitation associated with the potential spilling of untreated effluents, may put in danger the populations that need the water for their functions.

In another study, concerning the evaluation and possible impact of the migration of the Namibian population that lives in rural areas (80%) to the capital Windhoek (Namibia), water samples from Daan Viljoen, Avis and Goreangab dams were collected [219]. The presence of total coliform, *E. coli*, cultivable microorganisms, somatic coliphage and *C. perfringens* was tested, by membrane filtration and pour plate techniques. Water samples filtered with burlap, cotton and polyester, cheap materials, were also execute, to understand if they can significant reduce the microbiota concentration and reduce the potential danger from their consumption.

Cultivable microorganisms was significantly high in all unfiltered water: from Goreangab dam - 695,000 CFU / mL, Daan Viljoen dam - 257,750 CFU / mL and Avis dam - 3975 CFU / mL. Cultivable microorganisms was significantly lower in water filtered with polyester and cotton in Daan Viljoen and a reduction in cultivable microorganisms was also observed with all filtration system in Goreangab dam (no difference in the Avis dam).

Total coliforms were significantly high in unfiltered water from Goreangab dam (845,000 CFU / mL) and for *E. coli* in unfiltered water from Goreangab dam (±8500 CFU/ mL), but a significant reduction

was observed after filtration. There was a significant difference in the Daan Viljoen dam and no significant difference between unfiltered water and the filtered water from Avis dam.

The high value of total coliforms found in the Goreangab dam was explained by the use of this dam to store treated water that was reclaimed from households or reveal a potential ineffectiveness or malfunctioning of the treatment process employed. The high presence of *E. coli* in the Goreangab dam indicates water with fecal matter. In this study was again appointed the bad treatment of water as a potential factor in the microbiota concentration (and maybe disease- causing organisms such as bacteria, viruses, and parasites). There are several exogenous factors that may influence the microbiota values, and understanding them is essential to reduce or control the growth of pathogenic microorganisms. Nevertheless, is also important to do a critical evaluation of the treatments apply to sewage and verify if they are adapted to the waters in question.

In the waters samples from the present experimental work, the values of microorganisms never reach the concentrations registered in that study, a sign that the treatments and controls of the water of portuguese dams are efficient and well surveyed.

There was a significant decrease in the number of *C. perfringens* after filtration of water from Daan Viljoen dam, compared to the amounts found in the unfiltered water. The amount of *C. perfringens* found in unfiltered water from Goreangab dam was the same as that filtered wit burlap, but results showed a decrease of C*. perfringens* when filtered with cotton and polyester.

Somatic coliphages were high in all unfiltered water samples of all three dams, but filtration of the water samples reduced the number of the coliphages. Avis dam showed the higher coliphages contamination followed by Goreangab dam then Daan Viljoen dam. This showed that there were a high number of somatic coliphages which appeared to be present whenever the level of coliforms were higher.

In lake or ponds ecosystems the food chain is compose by phytoplankton and aquatic vegetation (primary producers), zooplankton (primary consumers, zooplankton) and fishes, amphibians, crustaceans and molluscs as the follower consumers. Plankton eat floating communities are very sensible to pollution. If anything happens in the water equilibrium and disturbs the overall quality, it can be detect calculating the biomass community [220] and by detecting the genera presence.

Phytoplankton are crucial to freshwater and marine ecosystems, but their composition varies frequently due to alterations in the chemical parameters of water. Some dams demonstrate a temporal well define pattern [221], that is though in the literature to be related in general with four constraints: light, temperature, stability of the water column and nutrients.

Cyanobacteria needs low light intensities, in terms of light, an advantage (against algae groups) in turbid dam waters. The temperature range is wide, but the growth rates are maximum when the temperature of 20 ºC is exceed (tropical climates favours cyanobacteria growth) [222]. Thermal stratification in water can be a factor in the phytoplankton prevailing and regulates the genera presented within the water column [195].

Cyanobacteria use a mechanism named “buoyancy”, in a stable water column, to be in the best water layer (best relation between light and CO_2). This mechanism allows them to migrate within the water column during water stratification [193]. Another advantage is their higher affinity for uptake

phosphorous and nitrogen, which allows them to sustain nutrient limitation conditions and survive longer that other phytoplankton [223].

The growing of photosynthetic microbes is expected in the water of an artificial lake generated by a dam, with sunlight incidence and inorganic nutrients available (especially phosphates); correlations can sometimes be found with environmental parameters, helping in predictions [224]. They constitute an integrant part of the ecosystem and sustainability of these waters' phytoplankton. An essential link in the eutrophic chain and part of feeding system (vital to zooplankton biomass), supplying nutrients. Also, release the oxygen that are needed for the respiration of higher taxa [90].

However, cyanobacteria are also a current problem in fresh and salted waters due to their exponential frequency through the formation of blooms episodes, especially due to eutrophication processes. With the phytoplankton development, the water becomes with abnormal physical characteristics, colored with shades of green, brown, yellow or reddish. Eutrophication also decreases the social value of waters and aesthetic enjoyment and health problems may occur, since eutrophic conditions interfere with the efficiency of drinking water treatments [9,226]. The eutrophication is a natural and global phenomenon, but can be further promoted by anthropogenic actions, in the form of runoff and sewage [93]**,** which is called "cultural eutrophication" (human inputs of nutrients). Erosion and atmospheric deposition of nitrogen are other sources [225]**.**

The euphotic zone (upper layer where sunlight radiation can penetrate) decrease and the higher dissolved oxygen levels are closer to the surface, with an excessive growth of cyanobacteria. Bacteria depend on organisms decaying, and as the amount of oxygen rises, some organisms suffocate and serve as source of nutrients. The levels of microbiota are higher in this condition (cyanobacteria also excrete organic matter) [227].

The cyanobacterial blooms unbalance the aquatic environment equilibrium in many ways and can be a significant water quality problem. Accessorily, species also produced dangerous toxins not only to human but also to the general fauna, including the sub aquatic fauna and other microbiota; 25 to 75% of cyanobacterial blooms are toxic [228].

To remove cyanobacteria from the artificial pond is not an easy task. The reduction of the nutrients inputs helps, but is not an immediate solution, because lake sediments can also serve as a nutrient reservoir [225].

There are an evident growing level of information concerning the role of cyanobacteria, but there is still unclear some ecological impacts. Cyanobacteria are controversial microorganisms, being both a fundamental element for the ecosystem, sustaining superior taxa´s, but also dangerous one´s, forming blooms, that may produce toxins, with health effects to all forms of life in contact.

A methodology to detect cyanobacteria and green algae was drawn up and applied in the present work, aiming to assess insight of these particular microorganisms, due to their potential pathogenic activity and as an indicator of the water conditions, and the trophic state.

An original procedure, in the present work, based on standard operating methodology, was developed. That included the use of fragments of modified BG-13 Agar immersed on water samples, and incubation under day light at room temperature [181]. Microscopic preparations were mounted using special fixing and staining techniques (iodine, malaquite green and safranine staining).

Different rotations and times were tried preliminarily, but none appeared to be efficient. Cell of cyanobacteria are fragile, and even with low rotation values they could not maintain intact their structure.

The only feasible way to conserve the samples was by direct isolations. This proved to be the most competent procedure to obtain cells for microscopic visualization and characterization. From each bottled sample (avoiding stirring), one or two drops were extracted with a Pasteur pipette, and were placed on a microscope slide (previously bathed in alcohol and dried). The drops were spread with a plastic loop.

Different colorations techniques were used, but Victoria Blue with Giemsa, not given accurate results to differentiate the genus; Malachite Green with Lugol was a more laborious coloration but the distinction of genus was accomplished; finally the application of a single drop of safranine allowed the visualization in every sample.

The medium had proven to be successful in the growing of cyanobacteria, although it also allowed the growth of micro algae, the water samples quickly change color, revealed at naked eye. These modifications were accomplished by the development of the target microorganisms, after the addition of the agar, having proven to be able to provide the essential nutrients.

There was micro-algae in 21 samples (80.77%) and cyanobacteria in 18 samples (69.23%).

From cyanobacteria, there was evidence of the genus *Microcystis* in 12 samples (66.67%) (42.31% in the 26 samples) and the genus *Snowella* in one sample. The results showed that the frequency of potentially hazardous cyanobacteria is high and these finds stressed the need of having always in consideration the toxigenic cyanobacteria evaluation when quality of dam´s waters are under scrutiny. The occurrence of cyanobacteria blooms is increasing and studies to verify the meaning of the presence of cyanobacteria should keep ongoing.

Not all water samples revealed the presence of algae, which can be linked to the intrinsic constitution of the waters, without exogenous influence: meteorological conditions [229], nutrients in water [222], activity of zooplankton [230]. Even competition or relation with others microbial genera may be a possible explanation. Some lakes analyses detected the same cyanobacteria genus in the same season each year (e.g. *Microcystis* spp. in summer). In reservoirs this pattern may differ, due to discharges. E.g. in the Murray Darling River, cyanobacterial variations correlate with low discharge periods [231].

The most frequently found genera of cyanobacteria were agents having poli-cellular structures without filamentous forms. These results may not correspond to the original frequency in waters of the dams, since enrichment procedure implement in the laboratory may have influenced the proportion between the different genera in presence. The direct samples all reveal the presence of cyanobacteria and the procedure has proven to be efficient concerning the preservation and disclosure of many cyanobacteria species. When compared with the usual direct methods, the cellular growth allows to assess better defined and stable morphologies that are essential to microscopic discrimination of structure and final identification.

Single direct visualizations can be misleading, detections reveal cyanobacterial populations with one or several species, but even at the level of strains there is differences in the toxigenic capacity. Variations may occur in response to determinants variables. To distinguish based only visualization is not possible. Molecular techniques are important to estimate the presence of potential toxigenic microrganisms [232].

The disadvantage of the molecular techniques is the lack of information concerning the level of production of toxins. Microcystins or other cyanotoxins were not searched but it is important to a comment that, its direct assessment can provide a more accurate information in terms of risk analysis.

The most obvious disadvantage stems from the fact that the presumption of dangerousness during the detection of potentially toxigenic agents and not directly from the hazard. Complementing microscopic visualizations of the cyanobacteria genus with toxins detection and quantifications, fully evidences can be reached.

Among the multiple genus and species of cyanobacteria that have been found, there were some that are generally acknowledged as having potential toxigenic ability. It was clear that these potentially toxigenic genera were found very frequently (almost 42% of samples were positive). During its process of development or “blooming” they can excrete a wide range of toxic metabolites (microcystins, nodularins, alkaloids, aplysiatoxins, anatoxin-a, cylindrospermopsins, β-methylamino-L-alanine and saxitoxins) - all of them having potential severe consequences to human health.

To develop comprehensive assays for cyanobacteria identification and allowing direct toxin detection and quantification so that a full explanation for those risks can be determined is crucial to assess the safety of water retained in dams. Several factors are believed to interfere with this autotrophic bacterial, especially, available inorganic nutrients (polyphosphates, ammonia, potassium, iron, magnesia, calcium) and light. The consequence of human or animal exposition to these hazards are not entirely elucidated.

Detection of microbes that are potential cyanotoxins producers is a challengeable task due to the weak development of accurate methodologies. The development of professional skills to identify these agents requires special training and development of standardized procedures. Without capability is not easy to put in place adequate management systems to alert the possible conditions of risk or signals of its presence and its control. Preventive measures, avoiding water eutrophication are the best strategy to control cyanobacteria blooms, since the “curative” treatments applied to eutrophic waters are not 100% efficient.

Cyanobacteria are frequently found in fresh and marine waters, but the most abundant microorganisms are viruses. The roles of viruses are crucial to the ecological stability and control of the food web and microbiota present. They kill microbes, doing a natural selection and pressure in the microbial diversity and are a potential food source for protists [233].

The last chosen indicator was phages of an enteric human bacteria, due to the increasing virus investigation as waterborne pathogens and the importance of bacteriophages as indicators, for being relevant in order to predict enteric viruses contaminations and human fecal contaminations.

Most data concerning the incidence of phages in water environments are on somatic coliphages, because somatic coliphages are detectable by simple, inexpensive and rapid techniques, and the phages occur in large numbers in any water environment exposed to human or animal excreta. Phages have proven to be indirect valuable tools in research on viruses and have been projected as microbial indicators of water quality, as they share many fundamental properties with common human enteric viruses which pose a health risk [234].

Enteric bacteriophages correlations with enteric viruses are hard to make and are several times inconsistent [235]. The host bacteria was selected to limit possible confusions. *Shigella sonnei* is a specific bacteria present in the human intestinal tract and the presence of its bacteriophages are an evidence of possible fecal human contaminations, even if correlations with other microorganisms are not referred.

A straightforward methodology has been used to detect bacteriophages of human enteric bacteria by conventional methods, using plaque assays with susceptible selected host bacteria. The filtrate was put on the surface of the nutrient agar where the host bacteria has been previously spread. The visualizations of formed lytic plaques, after 24 hours of incubation, allows to quantify the phage presence and number (by inoculums dilutions), but after trying several times executing the selected procedure for bacteriophages, no detection was accomplished.

A possible explanation for the absence could be the time it takes to perform the analyse, because some samples were saved for months under cold temperatures that could affect the phages stability. Some studies reveal that enteric viruses can resist cold temperatures in the environment: enteroviruses for 4-6 months (frozen water); polioviruses, coxsackieviruses, echoviruses and Hepatitis A Virus for several months (in marine water and in groundwater) [236]. Another studies reflect that both enteric viruses and coliphages survive better and longer in cold temperatures (decay in higher temperatures) [235],[237].

Lack of a viable susceptible host strain was not a limitation as they growed without problems in the culture medium. Maybe bacteriophages from other strains or species were present but could not be detected.

Absence of bacteriophages from such a human fecal specific microorganism can be a positive sign that human effluents inflow are under the correct treatments to avoid fecal contaminations of basin. Only fecal contaminations from animals may be present in the samples with high values of *E.coli* and *Enterococcus* spp.. This method reveals that none bacteriophage of *Shigella sonnei* was present in the waters samples that were analysed. However, *Shigella sonnei* is not a universal intestine bacteria, being very rare on portuguese population.

Official limits stating the classification of the “water quality”, for each microorganism, are in place and were taken in consideration to decide the organization and the nature of the procedures applied to water sanitation. If the parameters used for water classification were only *E. coli* or total coliforms, great disparities were not detected. *E. coli* had one more sample with quality A2 (14 in 25) than coliforms did (and one less water classify as A1). What is more interesting is the results using only *E. coli* or *Enterococcus* spp., with the previous having 20 samples (from 26) with quality A1.

All the enumerate microbiota in consideration are taken simultaneously, in drinking water supply systems, and the evaluation is based on the parameter that reflects the lower quality. Comparing the different classifications, 10 samples showed contaminations compatible with the A1 ranking, 16 quality A2 and none had quality A3.

Using as single parameter *Enterococcus* spp. it can be concluded that most of the dams presented good quality and did not need special treatments while with *E. coli*, 12 samples would need treatments, besides the physical treatment and disinfection (water quality A3 require affinition chemical treatment) (Dec. Law n.º 306/2007, of 27 August).

It is not clear which parameter is more accurate to state the quality status of waters or what result is more trustful, taking these results in consideration. Both indicators are officially considered adequate to assess and classify the waters; there are extense scientific production appointing this [57, 62, 77, 179, 233, 237].

Enterococcus spp. have more strict limits and, in this work, its results do not show order values in the same scale as *E. coli*. A positive correlation was found between the two indicators (rho = 0.66, $p < 0.05$), by statistic analyse. A positive correlation shows that the indicators have the exact same tendency. A negative correlation was not anticipated because both these microorganisms have increased value when a fecal contamination is present; the “strength” of the correlation was the factor of study. The correlation value was positive, but not very high when compared with the correlations between *E. coli* and coliforms or with cultivable microorganisms.

The correlation ratio between coliforms and *Enterococcus* spp. was lower (rho = 0.53, $p < 0.05$). It had a lower value comparing to *E. coli*; this was expected because coliforms may be less correlated with fecal contaminations and shows the better correlation that *Enterococcus* spp. has with *E. coli* alone.

Other factors could explain this difference, like contaminations of animal origin, the temperature or pH of the waters, inflow discharges of urban or industrial effluents.

All of these achievements reflect the complexity of the models that are currently in practice to evaluate the microbiota of waters retained in dams. Many relevant microbial problems are not put in perspective when these waters are routinely evaluated, like the microbiota of the anaerobic or the aphotic zones of the water column and the microbes of the sediments. For safety issues, concerning utilization of those waters for consumption, it is important to have more than one single fecal indicator: together they elucidate better the “water quality” question. Each indicator has its own ecological reference and specific responses to environmental stress factors and a combination of both is the safest way to guarantee the correct conditions of water and avoid possible hazards and risks [63].

2.5. Conclusion

The strategic importance of dams´ water to sustain life on earth and all its ecosystems is unquestionable. In this work a research on microbial characteristics of surface waters from Portuguese dams was performed, involving several samples collected across the country to assess its quality. In general, the results showed that microbial characteristics were satisfactory.

Similar works are essential to assure the adequate protection of the populations health, avoiding their exposure to hazards coming with the water, whether intended for recreation, irrigation or drinking. The results allowed a discussion concerning the relevance of each elements of microbiota present in those waters.

The tests, which were applied, revealed to be quite practicable and efficient on the detection and enumeration of the major microbial groups (total cultivable microorganisms, coliforms*, E. coli*, *Enterococcus* spp.). A preliminary analyse, concerning the influences of geographic locations of sampling, the season, or even external contaminations, showed that these topics must be considered as critical.

Several factors may affect the results; some of them were commented in this work. The perspective behind that scenario, concerns to the fact that water's microbiota is not a steady, uniform or very predictable object; it is much more an every time changeable living mass that interacts with the environment in unimaginable ways.

The chosen indicators, fixed by the legislation, further reflect this, due to their own definition; they are microorganisms expected to predict the presence of others, having similar characteristics. It is not possible to say, with all confidence, that this really happen. Even so, positive correlations obtained in this work are an ensuring finding.

The microbiological control of water depends on the ability to verify the presence fecal pollution, which, allied to the improvements on the treatment and disinfection of water, help in the control of waterborne health risk around the world. Some outbreaks of waterborne diseases are still occurring worldwide, namely due to viruses (norovirus, rotavirus, Hepatitis A virus) and pathogenic bacteria of the enteric environment.

A special attention must be given to the cyanobacteria presence in freshwater, since with the increasing of blooms development, due to agricultural practices intensification, it is a growing problem.

The results obtained in the present work, showed a high frequency of potential microcystins producers in the samples. These preliminary results have been already presented in the 4º Iberic Congress of Cyanotoxins (annex 16), in which the importance of these cyanobacteria was much enhanced. Detection and quantification of cyanobacteria and its role on disturbing other organisms was a common topic, being also an opportunity to reflect about the protection of health.

Not only a complete investigation of cyanobacteria at the level of specie (or even strain) with direct visualizations, mass-spectrometry or molecular methods are needed, but an intensification of government measures to predict cyanobacteria presence in surface waters and a more worldwide divulgation about the involved problems are crucial for the future.

The presence and the role of microorganisms in freshwater collections are always being revisited; this work is preliminary approach and a contribution to the characterization of the microbiota.

Microbiota of waters is still a source of diseases and deaths, but understanding the causes and possible influences are key elements that will allow more efficient control programs. The possibility of direct detection of the pathogenic microorganisms, using molecular techniques, is a future solution, but still not much in practice.

This work showed how the monitoring of indicators microorganisms for water pollution and quality evaluation, could be low cost and feasible to perform, with the results opening more questions and further research to fully identify the species present along an extended period of time, with more variables evaluated (chemical factors, temperature, nutrients, within others).

Furthermore, the preservation of freshwater resources around the world, including those collected in dams, is one of the greatest challenges that humanity is facing nowadays, since it is “the life”, itself, that is under threat.

3. References

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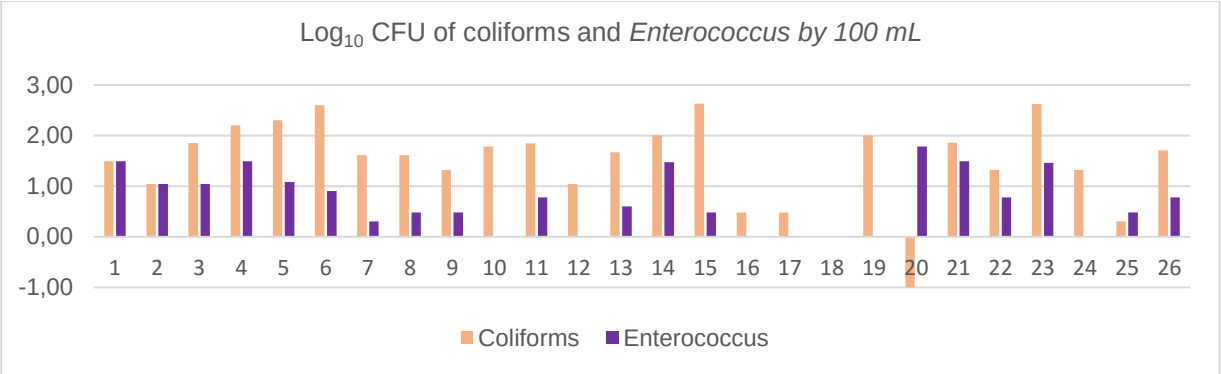
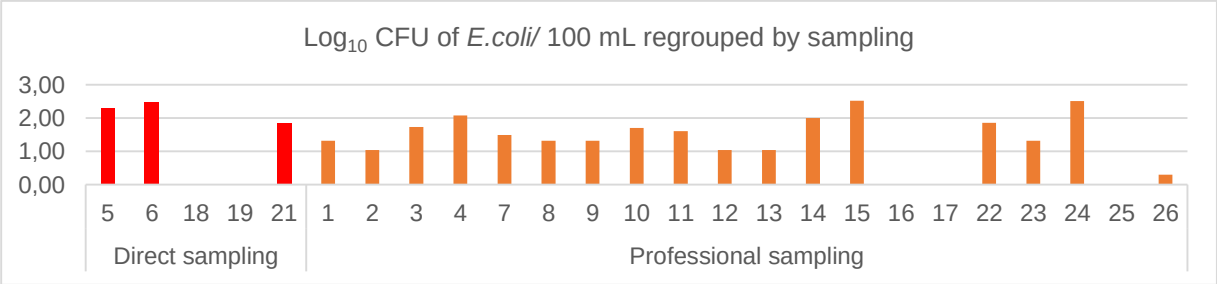
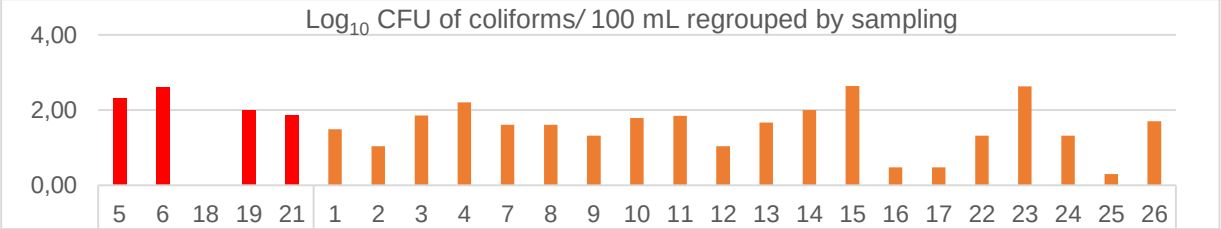
Annexes



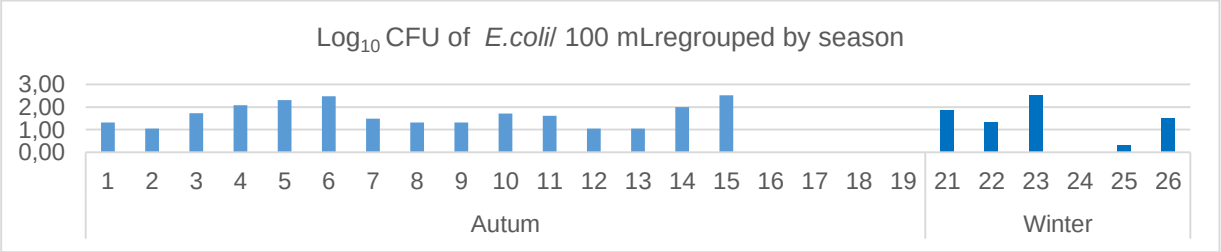
Annex 1 - Enumeration of both coliforms and Enterococcus spp. (CFU log_{10} values by 100 mL) in Portuguese dams' water (September 2014 to March 2015)



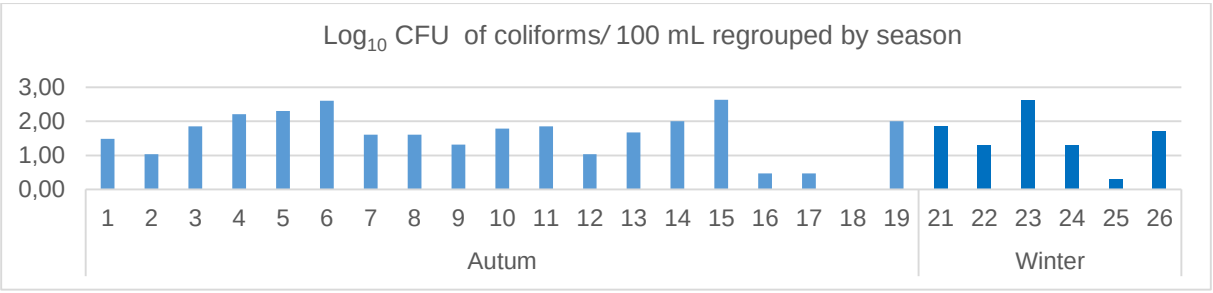
Annex 2 - Comparison of results obtained for E. coli (CFU log_{10} values by 100 mL) with "direct sampling" and samples collected by a "professional laboratory"



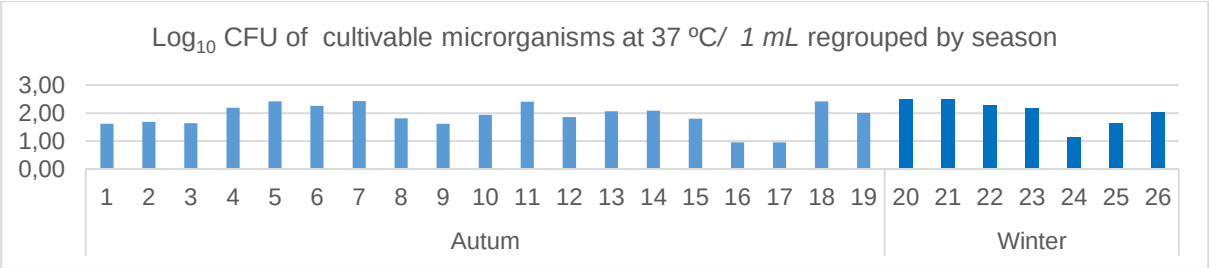
Annex 3 - Comparison of results obtained for coliforms (CFU log_{10} values by 100 mL) with "direct sampling" and samples collected by a "professional laboratory"



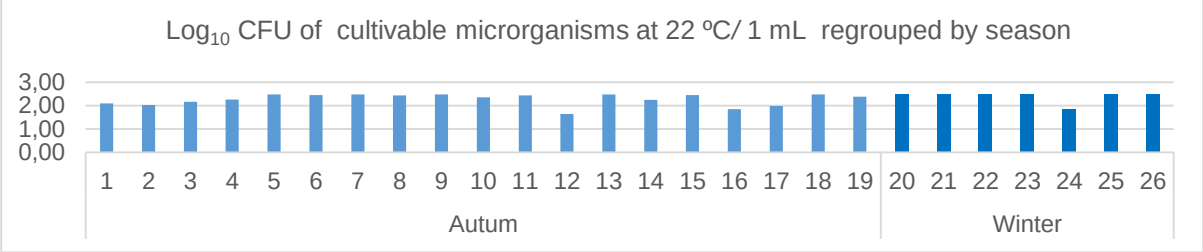
Annex 4 - Comparison of samples from different seasons (CFU log_{10} values by 100 mL) for *E. coli*



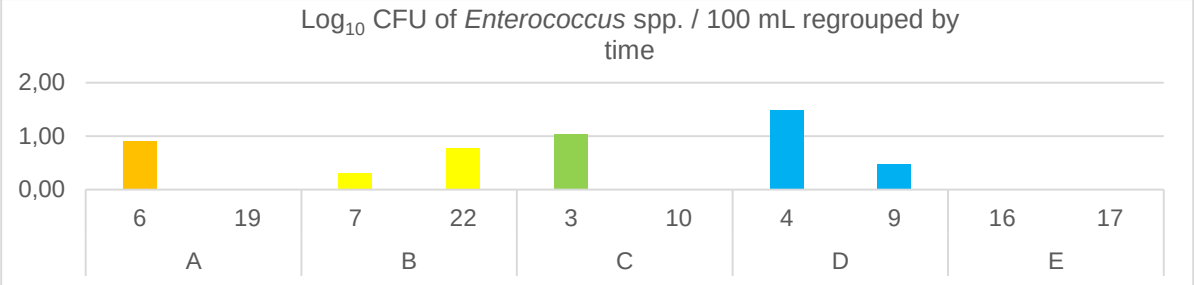
Annex 5 - Comparison of samples from different seasons (CFU log_{10} values by 100 mL) for coliforms



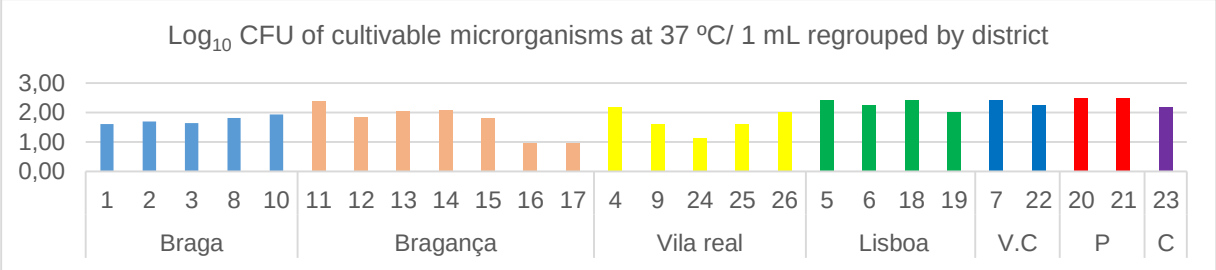
Annex 6 - Comparison of samples from different seasons (CFU log_{10} values by 1 mL) for cultivable microrganisms at 37 ºC



Annex 7 - Comparison of samples from different seasons (CFU log_{10} values by 1 mL) for cultivable microrganisms at 22 ºC

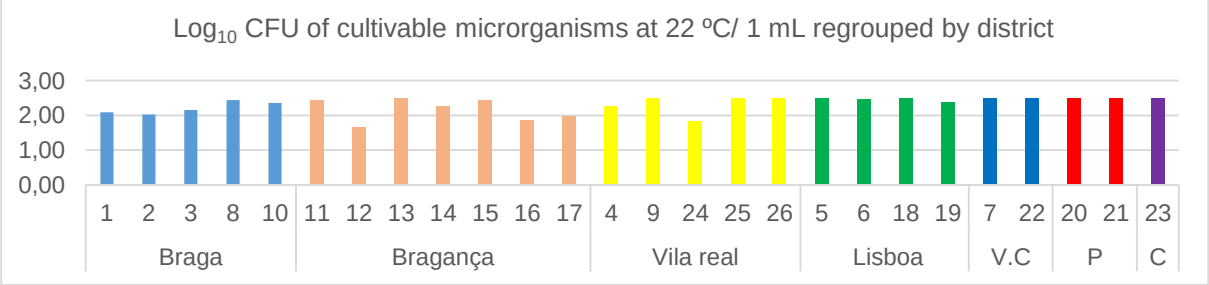


Annex 8 - Comparison of twice sampled waters from five dams for *Enterococcus* spp. (CFU log_{10} values by 100 mL)



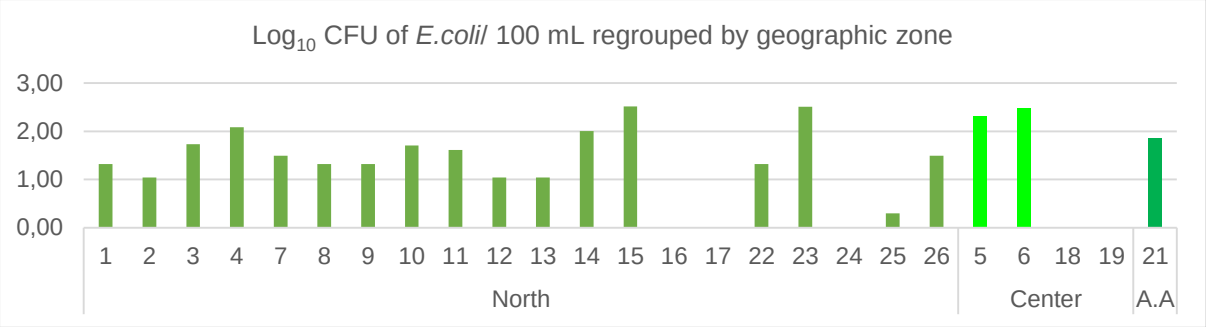
Legend: V.C- Viana do Castelo; P- District of Portalegre; C - District of Coimbra

Annex 9 - Comparison of samples coming from the different districts for cultivable microrganisms at 37 ºC (CFU log_{10} values by 1 mL)



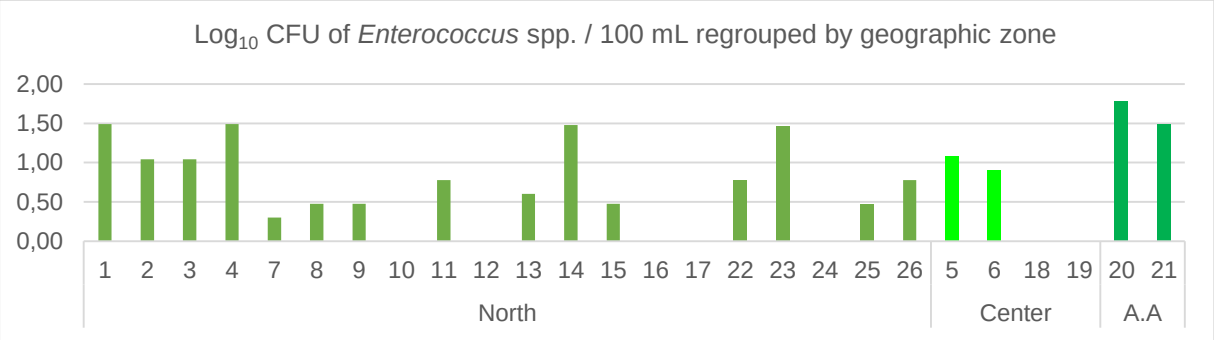
Legend: V.C- Viana do Castelo; P- District of Portalegre; C - District of Coimbra

Annex 10 - Comparison of samples coming from the different districts for cultivable microrganisms at 22 ºC (CFU log_{10} values by 1 mL)



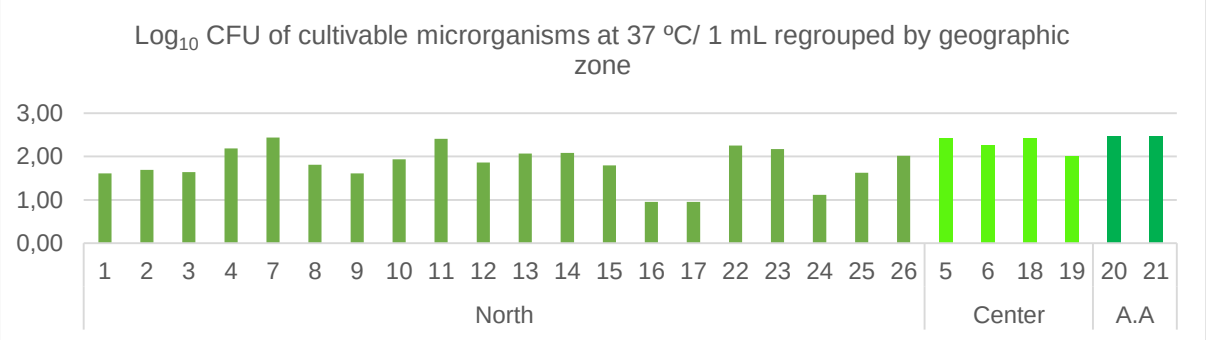
Legend: A.A – Alentejo zone

Annex 11 - Comparison between samples from different geographic zones for *E. coli* (CFU log_{10} values by 100 mL)



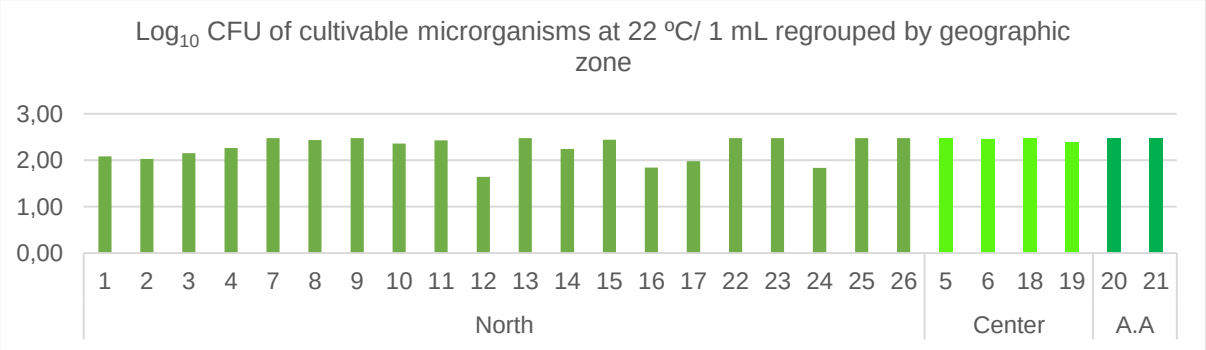
Legend: A.A – Alentejo zone

Annex 12 - Comparison between samples from different geographic zones for *Enterococcus* spp. (CFU log_{10} values by 100 mL)



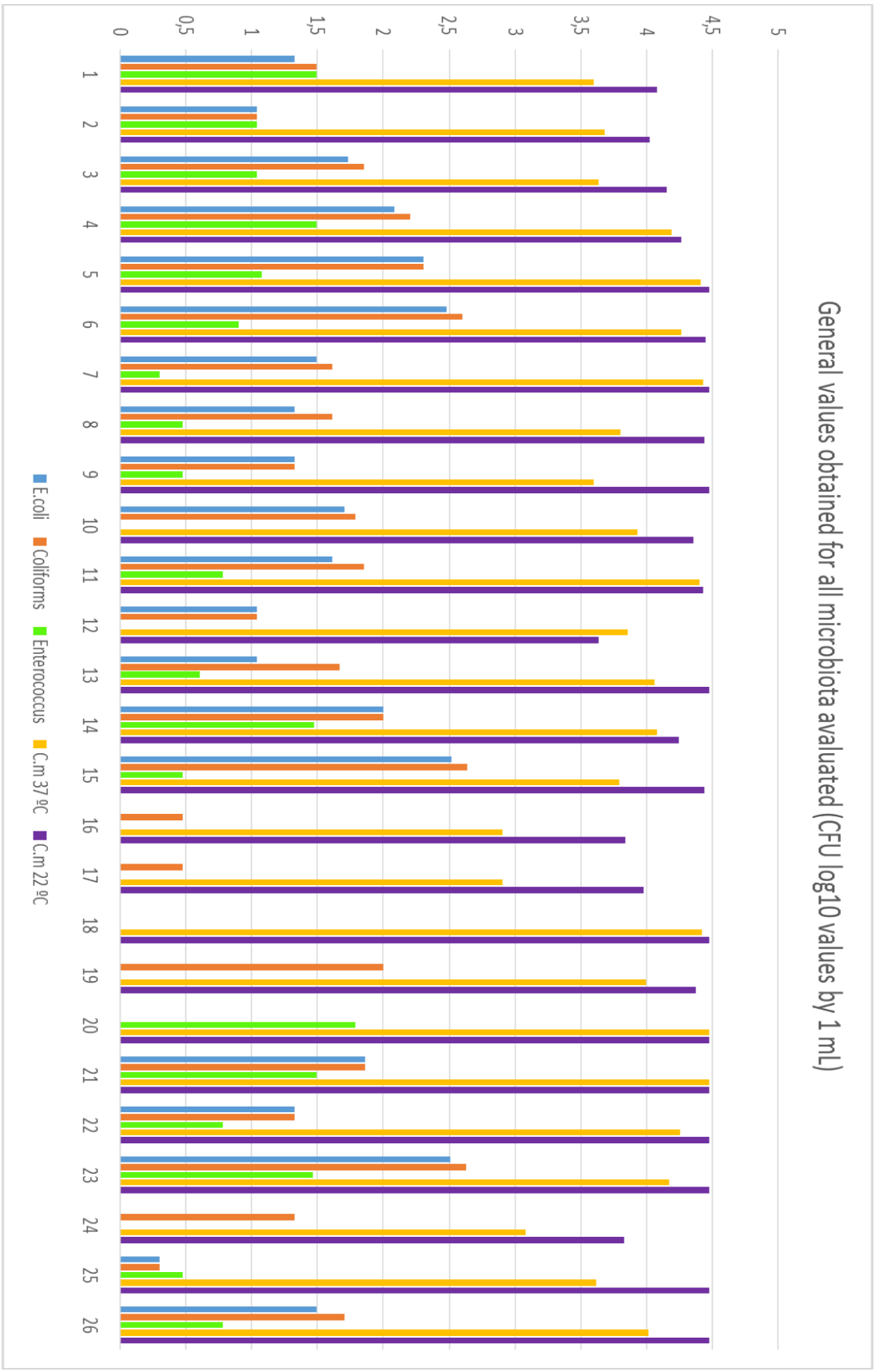
Legend: A.A – Alentejo zone

Annex 13 - Comparison between samples from different geographic zones for cultivable microrganisms at 37 ºC (CFU log_{10} values by 1 mL)



Legend: A.A – Alentejo zone

Annex 14 - Comparison between samples from different geographic zones for cultivable microrganisms at 22 ºC (CFU log_{10} values by 1 mL)



Annex 15 - General values obtained for all microbiota evaluated (CFU $\log_{10}$ values by 1 mL).

S1P4

Characterization of cyanobacteria in waters from Portuguese dams

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Cyanobacteria, or blue green algae, due to sharing characteristics with both algae and bacteria, are bacteria with a blue-green color from their capacity to photosynthesize (autotrophs). It is believe that they are responsible of providing food and oxygen for nearly all life on Earth, making up the bottom of the food web.
They occur worldwide (frequently in calm and rich nutrients water), and when optimal conditions are achieve they can form blooms, becoming the dominant organism with a possibility of reducing the water quality. Some species produce toxins (microcystins) which can harm both humans and animals. Depending on the affected organ in humans, they are refer by different names, but all share an absence of odor or taste, being a cause of gastrointestinal problems, headache, and even promote cancer.
The capacity of a massively development with the production of microcystins reveals the importance of detailed studies about their presence and activity in waters. Cyanobacteria in surface water is still a problem that lacks an ample investigation, to avoid the dangerous consequences from this microorganism.
In this work, 26 water samples from 19 dams in Portugal were analyse to identify the major genus of cyanobacteria present. Between autumn and winter of 2014-2015 a standard operating procedure, resourcing to the medium BG-13 modified, was apply. 18 samples (69.23%) reveal the presence of cyanobacteria, from which 12 samples (66.67%) had potential microcystins producers.
These results show a frequency of genera of potential pathogenic cyanobacteria, microcystins producers that may put in danger organisms expose to this water.

Keywords: Cyanobacteria, Microcystins, Dam

Annex 16 - Presentation (Poster) in the 4º Iberic Congress of Cyanotoxins, Lisbon, 08-10 July 2015. Abstract from the book of abstracts (page 22)