Assessing the microbiota of waters from Portuguese dams

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Abstract

Sustainable systems for drinking water supply are challengeable tasks. Dams are a very useful resource to provide water availability. Many physical, chemical and microbiological controls are needed to assure the eligibility for drinking of waters coming from dams.

The objective of this work was to perform a preliminary assessment concerning the microbial characteristic of Portuguese dams' waters. Microbial indicators of water quality were searched to achieve that goal.

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. Additionally, a methodology to detect bacteriophages, cyanobacteria and green algae was drawn up and applied in the present work.

This preliminary work presents the results of some microbial tests applied to 26 samples of water collected from 21 portuguese dams (seven different districts of the country) from September 2014 to March 2015.

The results allow different classifications, attending to official standards in use: 10 samples showed contaminations compatible with the A1 ranking, 16 quality A2 and none had quality A3.

Micro-algae were detected in 21 samples (80.77%) and cyanobacteria in 18 samples (69.23%). The most frequent genera of cyanobacteria were *Microcystis* (66.7%) and *Swonella* (5.6%) in the 18 samples.

Keywords: Dams' water, microbial indicators, cyanobacteria.

Introduction

Monitoring all the natural or pathogenic microorganisms that may be present in the water of dams, is not practicable and an efficient way to access water microbiota and safety. There are several factors that are able to affect the microbial populations of waters including exogenous anthropogenic contaminations or even agents coming from flora, fauna and soils.

The introduction of waterborne pathogens is, in a succinct way, due to the inflow of external contaminations, the frequency of those events, the persistence and transport of pathogen in the site, and its resistance to the applied treatments.

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 some pathogens.

Routine water microbiological analysis does not include the direct detection of pathogenic agents, attending to these reasons. Nevertheless, so that water may be considered safe, it must be free from pathogenic microbes. The conciliation of the two principles was met by testing for indicator bacteria [1].

To use an indicator is an indirect way of indirect assessing the pathogens, without detecting or quantifying them directly. When an indicator is found above a critical level, it can be inferred that the pathogens (cohabitant of its ecosystem), may be, or has been, present in the water. The presence of an indicator does not correspond, for sure, that pathogens are present, but it allows signalizing fecal materials in the water. Fecal indicators do not signalized the presence of water native pathogens like *Leptospira* spp. or *Legionella* spp.

In the water of the artificial lake generated by a dam, with sunlight incidence and availability of inorganic nutrients (especially phosphates), the growing of photosynthetic microbes is expected (cyanobacteria, micro-algae). Correlations can sometimes be found with environmental parameters, helping in predictions [2].

They constitute an integrant part of the ecosystem. Phytoplankton is an essential link in the eutrophic chain and part of the feeding system, vital to zooplankton biomass, supplying nutrients and releasing the oxygen that are needed for feeding and respiration of higher taxa. 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 the eutrophication processes.

There is an evident growing level of information concerning the role of cyanobacteria, but its global impacts in the ecosystems are still unclear. Cyanobacteria are controversial microorganisms, being a fundamental element for the ecosystem, sustaining superior taxa´s, but also dangerous one´s, forming blooms, that may produce toxins, threatening the health.

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

The characterization of the water microbiota was 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 significance.

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 was to perform a preliminary study concerning the microbial characteristic of Portuguese dam´s water. Indicators from water contamination and quality were searched for that.

Results were also used to verify if the sampling methodology and the season may have some influence in the results. Other microbial determinations were performed accessorily, namely: search of cyanobacteria and bacteriophages of human enteric bacteria.

Methods

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)

Twenty samples has been 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").

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. Seasonality, geographic origin of samples, comparison between two samples from the same dam (sample 16 and 17) and from the same dam at different sampling time was also evaluated.

In terms of microbial determinations, six different analytical procedures were executed: enumeration of total cultivable aerobic microorganisms at 22 ºC and 37 ºC, enumeration total coliforms, enumeration of *E. coli*, enumeration of *Enterococcus* spp., (using methodologies based on national and international standard procedures, to evaluate dams' water characteristics) 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.

Detection and quantification of cyanobacteria, 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 original procedure, based on standard operating methodology, was developed. Fragments of modified BG-13 Agar were immersed on water samples followed by incubation under day light at room temperature for a month [2]. Microscopic preparations were mounted, after incubation, using special fixing and staining techniques (iodine, malachite green and safranine dyes), followed by microscopic observation. Cell enumeration and cyanotoxins quantifications were not tried, although some preliminary cultures were essayed using plating account in modified "BG-13 Agar" [3].

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

Optic microscopic visualizations were executed to detect and identify algae and cyanobacteria, till the genus taxa, 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 [4]. Accessorily, other confirmations were performed. Some guides were used for that, including digital libraries of images assessable by internet [5]–[11].

The relative frequency of toxigenic genera was established in each positive sample, because cyanobacteria are more relevant when associate with toxins productions [12].

The following procedure was executed to detect human enteric bacteriophage: 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 24h, 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 h 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 h 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.

The results that were obtained with 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.

Statistical tests were applied to the obtained data, using a free software environment for statistical computing - "R Project for Statistical Computing" [13]. 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".

Results and Discussion

The general quality of Portuguese dam´s waters, attending to the results obtain on the present study, is quite satisfactory. 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.

Generally an aquatic biome is defined taken in consideration the amount of solved salts, being freshwater when the concentration of salt is under 1% (rivers, lakes). The microbiota found in these waters suffers an ecological pressure due to the temperature, pH, flow rate, light intensity and nutrients [14].

The waters of dams are colonized by typical native microbiotas that are also quite variable with the geographic location of the artificial lake and the longitudinal and vertical zonation of each specific water column [16 – 18]. In some seasons (especially in summer), a thermal stratification may occur.

The point where sampling collection took place could have affected the results as explain before, so the mean values of the results of total cultivable microorganisms were compared to verify if that collecting point had systematically different values. Higher levels of cultivable microorganisms were detected in samples collected directly in the littoral zone of dams (cultivable microorganisms at 37 ºC with statistical evidences).

These events may be due to the proximity of the benthic environment that has 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 [18], like fecal contaminations.

Samples collected in the winter season, showed an increase of the mean values of *Enterococcus* spp. and total cultivable microorganism; cultivable microorganisms at 22 ºC showed a positive correlation. *Enterococcus* spp. values were expectable since *Enterococcus* spp. can resist more extreme conditions [19 - 20]; it was a very dry season [21].

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

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 coming from "North" zone were professionally collected.

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 closer values to those observed in the samples coming from the "Alto Alentejo" zone for both cultivable microorganisms.

"Alto Alentejo" region had the highest values for coliforms, *E. coli* and *Enterococcus* spp.. All the detected coliforms were *E.coli*, confirming fecal contaminations.

The "North" region reveal the lowest mean values for coliforms while from the "Center" region *E. coli* and *Enterococcus* spp. had the lowest mean value. This zone were probably more expose to environmental contaminations

Microorganisms used as fecal indicators revealed low levels of contamination in general.

Bacteriophage, also called phage (or bacterial virus), are a group of viruses that infect bacteria, having, most of them, 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. None has been detected in the present work.

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.

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.

The ensemble of the results support the idea that physical stratification of the water column is a factor influencing the microbial burden of these surface waters. The specific determinant factors were not studied, but it seems clear that is quite variable with geographic location of the dam.

All the enumerate microbiota in water plans are take simultaneously in consideration and the evaluation is based in the parameter that reflects the lower quality. 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.

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).

Taking these results in consideration, it is not clear to say which parameter is more accurate to state the quality status of waters or what result is more trustful. Both indicators are officially considered adequate to assess and classify the waters; there several articles appointing this [1, 25].

Micro-algae were detected in 21 samples (80.77%) and cyanobacteria in 18 samples (69.23%). Predominant genera were *Microcystis* (66.7%) and *Swonella* (5.6%) in the 18 samples. Not all water samples revealed the presence of micro-algae, which can be linked to the intrinsic chemical constitution of the waters, without exogenous influence.

Among the multiple genera 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). A wide range of toxic metabolites can be excreted (microcystins, nodularins, alkaloids, aplysiatoxins, anatoxin-a, cylindrospermopsins, β-methylamino-L-alanine and saxitoxins) during their process of development, or “blooming” - all of them having potential severe consequences to human health.

To assess the safety of water retained in dams is crucial 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.

Several factors are believed to interfere with this autotrophic bacterial, especially, available inorganic nutrients (polyphosphates, ammonia, potassium, iron, magnesia, calcium) and light. Human or animal exposition to these hazards is not entirely elucidated.

Detection of microbes that are potential cyanotoxins producers is a challengeable task due to weak development of accurate methodologies. The development of professional skills to identify these agents requires special training and development of standardized procedures. It is not easy to put in place adequate management systems, without capability, 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.

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. It is important to have more than one single fecal indicator, for safety issues, concerning utilization of those waters for consumption: together they elucidate better the “water quality” question.

Each indicator has its own ecological characteristics 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 risk.

The results showed that the frequency of potentially hazardous cyanobacteria is high and these finds stressed the need for having always in consideration the toxigenic cyanobacteria as a parameter that must be put in perspective, whenever the evaluation of the quality and safety of dams’ waters are under scrutiny. The occurrence of cyanobacteria blooms is increasing; studies aiming to verify the presence of cyanobacteria should be keep ongoing, and more accurate methodologies are also needed.

Conclusions and future work

The strategic importance of dams´ waters 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. The results showed that microbial characteristics were satisfactory.

Similar works are essential to assure the adequate protection of the population’s 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 virus (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 producer´s in the samples. These preliminary results have been already presented in the 4º Iberic Congress of Cyanotoxins, 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´s 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 dam´s, is one of the greatest challenges that humanity is facing nowadays, since it is “the life”, itself, that is under threat.

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