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BIOTECHNOLOGY

Insights into multiple use water enterobacteria from the Brazilian semi-arid: the first approach by pyrosequencing

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ABSTRACT. Thousands of people living in semi-arid regions face problems of drought and loss of water quality. In addition, high incidence of acute diarrheal diseases related to water consumption has been responsible for a high number of deaths and high economic costs for human health. Many of the diseases can be caused by the presence of enterobacteria in reservoirs that serve for multiple purposes. This study aimed to confirm the presence of potentially harmful bacteria, which was highlighted in other articles, and to reveal non-identified genera by culture-dependent methods and pyrosequencing. Twenty-three genera of the Enterobacteriaceae family were detected, with emphasis on *Escherichia* genus and confirmation of the presence of species such as *Salmonella enterica* and *Enterobacter cloacae*. The abundance of heterotrophic prokaryotes and the physical and chemical data show an expected average for this type of environment due to the numbers historically presented in previous articles. The unprecedented detection of the presence of some potentially pathogenic species can alert and raise awareness of the populations that use stored water in the semi-arid regions. Consequently, as a result of the peculiar characteristics of reservoirs under this climate influence, there is a cosmopolitanism of enterobacteria that may be related to the alarming numbers of infections from Waterborne Diseases.

Keywords: acute diarrheal diseases; metagenome; pyrosequencing.

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Introduction

Arid and semi-arid regions present a series of socioeconomic problems that are also triggered by the environmental problem of water scarcity. In these places, the irregularity of rains cause periods of drought up to six months per year. Generally, part of the rainwater is drained by impermeable soils, while another portion evaporates rapidly under high temperatures and low relative humidity. According to Fontes, Zucchi, Medeiros, and Azevedo (2015), these characteristics also contribute to the salinization of surface waters and consequently to the loss of quality.

Climatic variations accentuated by periods of drought, as well as influences of urban development and the use of pesticides affect water quality and its availability. In addition, breeding of animals in the river vicinities and reservoir margins completes this common scenario in the Brazilian semi-arid region. Man-made reservoirs are the main water sources for human consumption and domestic purposes. Furthermore, many of these water supplies are destined to multiple uses such as electricity generation, irrigation and recreation (Sodré-Neto & Araújo, 2008).

These ecosystems, due to the interference of the mentioned factors, and the pollution resulting from the anthropic activity, are favorable to the microbial growth, even for those that play important roles in the functioning of the ecosystem, which contributes to the development of potential opportunistic pathogens (World Health Organization (WHO), 2017). As cosmopolitan organisms, the enterobacteria present in water for human consumption, whether native or allochthonous, may be related to a series of infections in the digestive, respiratory and urinary tracts, as well as bacteremia and skin infections (Pindi, Ashwitha, Sandhya, Swapna, Ambika, & Yadav, 2013).

In Brazil, the study from Rufino, Gracie, Sena, Freitas, and Barcellos (2016) evidenced more than 4 million reported cases of diarrhea, including hundreds of consequent deaths. Most of them in semi-arid region, which are possibly related to the water supply crisis worsened by the drought periods. In the region supplied by the waters of the studied drainage basin, Nascimento, Araújo, Nascimento, and Sodré-Neto (2013) address the matter of Acute Diarrheal Diseases (ADD), which occur mainly in children under ten years old, based on public health database.

This research aimed to confirm the presence of potentially harmful bacteria presented in other articles, and to reveal non-identified genera by culture-dependent methods in environmental sample by means of pyrosequencing. Accordingly, the importance of this study is justified by the use of metagenomic pyrosequencing in environmental samples due to the lack of molecular studies on freshwater in the Brazilian semi-arid region, besides the elucidation of the human pathogenic enterobacteria presence not yet described in previous researches.

Material and methods

Sampling

Armando Ribeiro Gonçalves Dam is a eutrophic reservoir of multiple uses in a Brazilian semi-arid region (05°67’ S; 36°88’ W) with great water storage capacity (2.4 billion m^3), and it is responsible for the water supply of 500 thousand people approximately. Measurable abiotic variables and quantification of prokaryotic organisms in the sampling site were estimated and analyzed between March 2010 and March 2012. Minimum, maximum and mean values are shown in Table 1.

A multiparametric probe (YSU 6820 V2) was used in situ to measure the limnological variables like water temperature, dissolved oxygen (DO) and pH. Water transparency was estimated using a Secchi disk (30 cm), and Total Phosphorus (TP) concentrations were estimated according to Valderrama (1981) and American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF) (1998).

Samples (20 mL) for prokaryotic heterotrophic abundance were fixed with buffered formalin (final concentration 2%; pH 7.4). Subsamples (2.0 mL) were filtered in a black polycarbonate membrane filter (Millipore®, GTBP; 0.2 μm pore size), using a support filter (Poretics®; 0.45 μm pore size). The abundance was enumerated by epifluorescence microscopy (Olympus BX41; dichroic mirror DM500; excitement filter BP460-490 and barrier filter BA520IF) of acridine-orange-stained cells (Hobbie, Daley, & Jasper, 1977). For each filter, at least 300 bacterial cells were counted in 15 to 20 microscopic fields.

DNA extraction and metagenomics

To perform the metagenomic analysis, in March 2012 a single and specific sample of 100 L from surface water was collected, and then filtered using plankton nets (68 μm and 20 μm meshes) for the removal of larger organisms. The concentrated sample in the 10 μm plankton net was kept in a sterile amber bottle (1 L), on ice, until the arrival at the laboratory, and then processed within 3 hours after collection.

DNA extraction was carried out using the Power Water® DNA Isolation kit (MOBIO Laboratories, Inc. CA, USA) according to the manufacturer’s instructions. After extraction, the DNA presence was confirmed by electrophoresis on 0.7% agarose gel, stained with SYBR Green. The photodocumentation of gels was made using the system L-Pix Ex (Loccus Biotechnology), and the DNA quantification (ng μl^{-1}) was performed using a NanoVue™ Plus Spectrophotometer.

After quantification, the DNA was sent to the National Laboratory of Scientific Computation (LNCC) – Petrópolis-RJ - where the pyrosequencing was performed by the platform 454 (ROCHE ®). Metagenomic sequencing files in FASTA format were transferred to the MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology) server (Meyer et al., 2008; Wilke et al., 2015) for taxonomic and functional microbial analysis.

Metagenome sequences, with an average size of 378 base pairs, were processed in the MG-RAST by means of standard criteria of stringency, at least 15 bp alignment length, 60% similarity and e-value 1e-5. 16S ribosomal RNA (rRNA) genes were identified by comparison with the RDP (Ribosomal Database Project) (Cole et al., 2009) and there was no duplicate artificial sequence production.

Results and discussion

The data obtained from Armando Ribeiro Gonçalves Dam study were the characteristics of the eutrophic aquatic systems from the Brazilian semi-arid, mainly of the drainage basin in which the studied environment is the main reservoir. In rainfall events, prokaryotic populations commonly increase due to both nutrient charge and the entry of allochthonous microorganisms (Sodré-Neto, Seleghim, Araújo, & Blaha, 2017). In the last years, many evidences suggest a relationship between the microbial quality of water reservoir and the occurrence of ADD (Nascimento et al., 2013; Rufino et al., 2016).

The physical, chemical and biological data (Table 1) such as temperature and pH, demonstrate a relative stability of fluctuations, with or without rain. Despite oscillations between minimum and maximum values, the TP and DO concentrations remained within the average found frequently in the reservoir. Other researchers have made similar observations in this same environment (Sodré-Neto & Araújo, 2008; Costa, Cunha, Panosso, Araújo, Melo, & Sant'anna, 2009; Nascimento & Araújo, 2013; Nascimento, Maia, & Araújo, 2016). Short numerical variations of measured environmental variables in the last 10 years may be related to the low climatic variability and to the prevalence of high and regular temperature throughout each year.

Table 1. Environmental variables (minimum, maximum and mean values) in sampling site between March 2012 and March 2012.

Variable	Mean	Min – Max
Total phosphorus (μg L^{-1})	66.27	29-105
Temperature (°C)	28.86	27.6-31.7
pH	8.89	8-9.64
Dissolved oxygen (mg L^{-1})	4.99	1.8-9.2
Secchi depth (m)	0.82	0.6-1.1
Prokaryotic heterotrophic density (10^7 Org mL^{-1})	2.06	1.52-3.38

Regardless of environmental conditions influenced by hydrographic factors, numerous microbial populations were observed, probably due to the influence of the stable factors like temperature and high nutrient concentrations. Warmer temperatures may have direct impact on pathogens survival rate outside of its hosts and the outbreaks of waterborne diseases (Morand, Owers, Waret-Szkuta, Mcintyre, & Matthew, 2013).

Several studies on aquatic communities and the presence of opportunistic pathogens in Brazilian semiarid reservoirs are investigations that address the eutrophication and ecology of these ecosystems. Their emphasis are usually upon interactions between communities and environmental variables, using quantification and identification techniques based on cultivable organisms (Bouvy, Falcão, Marinho, Pagano, & Moura, 2000; Costa et al., 2009; Moura, Dantas, Oliveira, & Bittencourt-Oliveira, 2011; Nascimento & Araújo, 2013; Nascimento et al., 2016).

High Prokaryotic Heterotrophic density (10^7 Org mL^{-1}) in Armando Ribeiro Gonçalves Dam and its historical data, sign that there is still much to be understood about microbial diversity of tropical reservoirs, mainly due to the small number of molecular approaches in studies developed in this region. Thus, culture-independent methods to study microorganisms are increasingly necessary to supplement information acquired through traditional research. Molecular methods based on the sequencing of the genetic material, since the 90's (Ward, Weller, & Bateson, 1990), help the population and functional diversity estimations to become more efficient when compared to the classic techniques, despite the importance of these when it comes to the presence of specific organisms for quality control (Vaz-Moreira, Egas, Nunes, & Manaia, 2011).

The entry of allochthonous microorganisms, inorganic and organic matter to reservoirs, effluent from anthropic activities, as well as the growth of autochthonous microbial communities, constitute some of the environmental problems addressed in recent years as the main concern related to the loss of water quality. These aspects influence the progress of researches carried out in different regions of the planet, both in the search for possible solutions to address scarcity and loss of water quality (Zhou, Khu, Xi, Su, Hao, Wu, & Huo, 2014; Mekonnen, Pahlow, Aldaya, Zarate, & Hoekstra, 2015), and in order to characterize microbial communities by conventional methods (Charity, Abanobi Okwouma, & Emeka, 2012; Sellami et al., 2012) or even by DNA molecular analyses such as Pyrosequencing analysis (Hong, Hwang, Ling, Andersen, Lechevallier, & Liu, 2010), Illumina-based (Degnan & Ochman, 2012), and PCR-DGGE (Sodré-Neto et al., 2017).

Data from pyrosequencing DNA analyses of microbial water communities, unprecedented for the researched Armando Ribeiro Gonçalves Dam ecosystem, revealed 253,800 DNA sequences and confirmed the presence of prokaryotes that may affect water quality and consequently the health of users. In this report, we highlight the occurrence of Gammaproteobacteria Class, which is commonly related to waterborne diseases, with emphasis on the prevalence of Enterobacteriaceae and Pseudomonadaceae families.

A total of 23 genus and three candidates to genus of Enterobacteriaceae family were revealed in this study. This family represented 13% of Gammaproteobacteria class, 3% of Proteobacteria phylum, and 0.8% of all detected Bacteria (Figure 1).

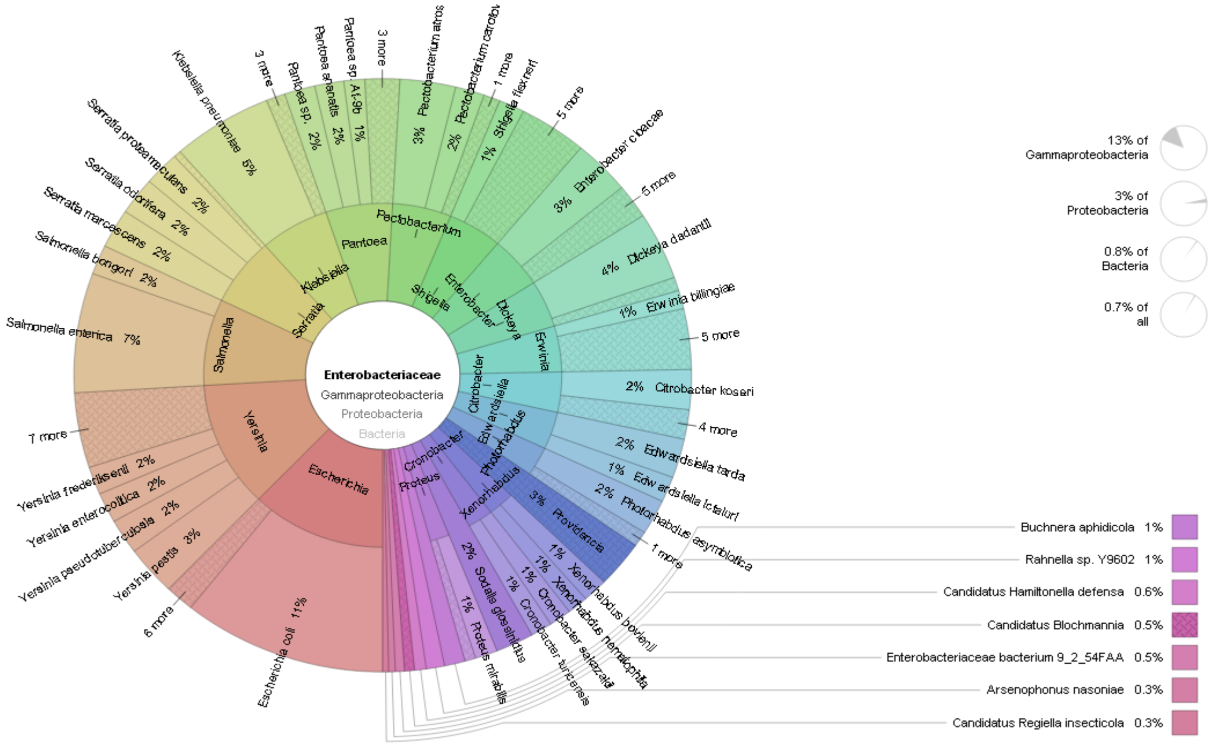

Figure 1. *Enterobacteriaceae* revealed by pyrosequencing 454. Source: MG-RAST metagenomic analysis server.

Our analyses also reinforce the expressiveness of enterobacteria living in soil, plants and water, as well as associated with other organisms sharing the same environment with the most frequent primary or opportunistic human pathogens, besides plant pathogens as *Erwinia* genus (Cellini et al., 2018), invertebrate pathogens as *Photorhabdus* genus (Peralta & Palma, 2017), and aquatic vertebrates pathogens as *Providencia* and *Edwardsiella* genera (Abdel-Latif & Sedeek, 2017).

The presence of enterobacteria, other than *Acinetobacter* and *Chromobacterium,* has been reported in the same aquatic ecosystem. Among the species detected by culture-dependent methods, *Citrobacter diversus, Enterobacter agglomerans, Enterobacter spp., Proteus myxofaciems, Proteus penneri* and *Serratia spp.* are opportunistic pathogens (Nascimento & Araújo, 2013).

Recently, Nascimento et al. (2016) also emphasized the predominance of enterobacteria and *E. coli* from sewage and wastewater. In addition to this problem, Nascimento and Araujo (2014) highlighted a possible presence of pathogens and multi-resistant bacterial strains in their systematic review.

The current study confirmed and estimated quantitatively these species, in addition to other viable but non-culturable bacteria. *Salmonella*, *Yersinia* and *Escherichia* genera represented about 25% of the detected enterobacteria, and some of them are directly related to the ADD that remain harming people who use the waters of semi-arid reservoirs. In turn, *Escherichia coli* (88% of *Escherichia* genus) accounted 11% of enterobacteria (Figure 2). The large number of this thermotolerant coliform may be associated with fecal pollution from diffuse sources added to high and regular temperature aforementioned.

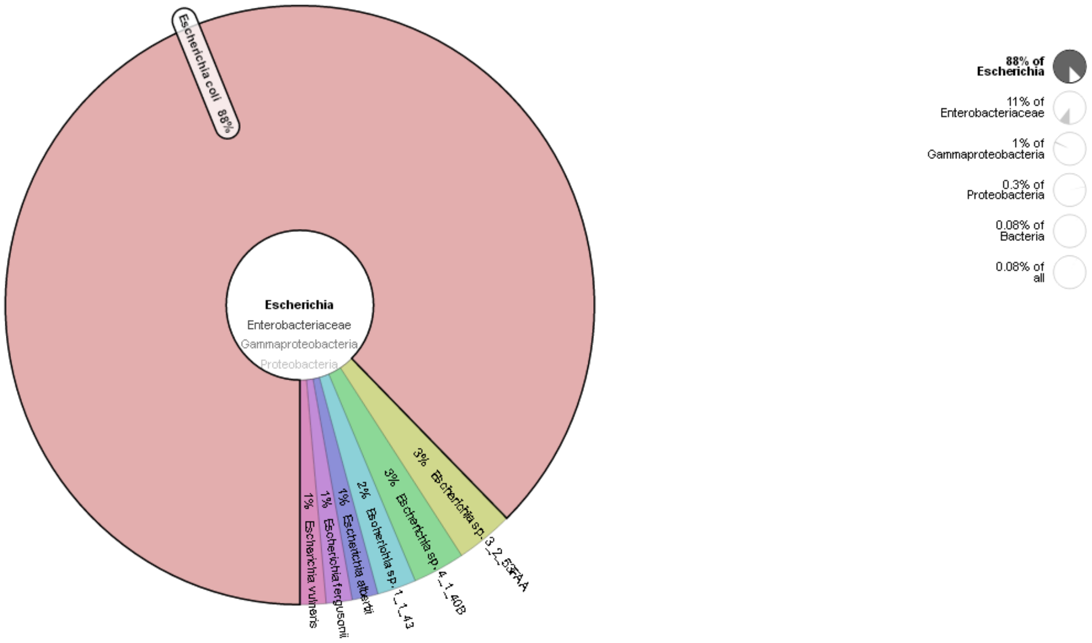

Figure 2. *Escherichia* genus revealed by pyrosequencing 454. Source: MG-RAST metagenomic analysis server.

According to Staradumskyte and Paulauskas (2012), most coliform bacteria do not cause diseases, however, some outbreaks of diseases are caused by *E. coli* 0157:H7, which is a strain found in cattle, pigs, and chickens. These are the most common animals in the proximity of the researched reservoir. Another problem is the contamination of natural environments with antibiotic resistant *E. coli* strains, and its impact on economic and health aspects (El Rahman, El-Wafa, & Ibrahim, 2015).

Salloto et al. (2012) reported that 5.7% of all infectious diseases on the planet are related to the use of low-quality water. In developing countries, there is an estimate that 80% of all diseases and more than a third of deaths are caused by the consumption of contaminated water (Dhanya, Anitha, & Dhanashree, 2013). ADD are more common in children, but they also affect adults. This type of infectious disease is still responsible for the mortality of many people around the world and it is one of the major causes of morbidity, even in developed countries (Façanha & Pinheiro, 2005). The difficulty of controlling the presence of potential pathogens in water for human consumption reinforces the need to characterize the relative abundance and diversity of microbes in the search for understanding the factors that govern the dynamic of these communities (Navarro-Noya, Suárez-Arriaga, Rojas-Valdez, Montoya-Ciriaco, Gómez-Acata, Fernández-Luqueño, & Dendooven, 2013).

Culture-independent methods to study microorganisms are increasingly necessary to supplement the information acquired through traditional research. Molecular methods based on the sequencing of the genetic material, since the 90's (Ward et al., 1990), help in population and functional diversity estimations and become more efficient when compared to the classic techniques, despite the importance of these when it comes to the presence of specific organisms for quality control (Vaz-Moreira et al., 2011). Thus, our pyrosequencing environmental DNA approach show new opportunities to access the prokaryotes at Armando Ribeiro Gonçalves Dam, in order to improve the management of water quality in these areas. This is a very important issue, since water storage is destined to multiple purposes, including the direct human consumption. The social context of this region, especially around the reservoirs, leads to a direct ingestion of this water, without treatment or microbiological analyses.

Conclusion

The use of molecular techniques such as DNA pyrosequencing opens new horizons for a complement to classical culture-dependent studies on the occurrence of microorganisms in water for human consumption.

The molecular analyses in Armando Ribeiro Gonçalves Dam contributed to the elucidation of some issues such as the presence of a large part of the non-cultivable microbial diversity, including potential human pathogens.

The obtained results may be an indicative of water contamination in Armando Ribeiro Gonçalves Dam, and they can be a starting point for further investigations on microbial communities and waterborne diseases, since they revealed for the first time the presence of uncultivable pathogens in water.

In these semi-arid regions, microorganisms are usually found in large numbers and may contribute to maintain the high incidence of ADD, besides the enhancement of health risks due to climatic, environmental and anthropogenic conditions.

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