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Limno-chemical and microbiology aspects in Uranium Pit Mine Lake (Osamu Utsumi), in Antas and Bortolan reservoirs under the influence of effluent Ore Treatment Unit, Caldas - Minas Gerais State, Brazil

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ABSTRACT

Due to high natural radioactivity there in Poços de Caldas Plateau (Minas Gerais State, Brazil) and the existence of the first uranium mine in Brazil (Pit Mine Osamu Utsumi - Mineral Treatment Unit/Brazilian Nuclear Industries, MTU/BNI), which is characterized by an open-pit mine presents as increased environmental liability the formation of acid mine drainage, this study was conducted to evaluate the limno-chemicals and microbialogy aspects (protozooplankton and bacterioplankton) belonging to uranium pit mine lake (PM) and evaluate the possible effects of acid effluents treated and discharged by MTU/BNI in Antas reservoir-AR and downstream of this, the Bortolan reservoir-BR. Besides the realization of abiotic and microbiology analysis of protozooplankton and bacterioplankton; was held standardization and deployment of the Fluorescence "In Situ" Hybridization (FISH) technical using oligonucleotide probes for extremophile Archaea and Bacteria. According to the results, the PM showed the highest values for the chemical variables, lower pH values, lower protozooplankton density, however, protozooplanktonic high biomass showing the presence of tolerant species in this extreme environment. Antas and Bortolan reservoirs showed differences in the abiotic and biotic variables, AR showed suffer greater interference of acid effluents released at P41point and downstream of this at P14 point, lower protozooplankton biomass, lower bacterial density and pollution characteristics of inorganic sources. Using the FISH technique standard in this study to water bodies evaluated, it was possible to detect the presence of the extremophile bacteria of the Archaea domain in the three water bodies. The results of this study contribute to the knowledge of the pit mine lakes limnology which have become a major concern due to increased mining in the open.

1. INTRODUCTION

One of the most serious environmental problems caused by mining industry is acid mine drainage (AMD) and the resulting acidification of water resources near the area. The AMD is characterized as a metal-containing acidic water released from the oxidation of sulphide minerals present metal sulfides, which is catalyzed by oxidizing acidophilic microorganisms iron and sulfur. [1] The presence of several bacterial species, including prokaryotes such chemolithotrophic *Acidithiobacillus* spp. and *Leptospirillum* spp., have been reported in mining environments. The fundamental condition for the occurrence of bacterial leaching and the AMD is the presence of metal sulfides, such as pyrite (FeS_2) associated with the crude ore, as occurs in cells of sterile generated by Osamu Utsumi uranium mine, Caldas city (Minas Gerais, Brazil) [2].

In the region of the headwaters of the Antas reservoir (AR), begins the Sub-Basin of the Antas River which is the largest and main hydrographic network Poços de Caldas plateau in Minas Gerais state. Antas reservoir was built to supply water to the Ore Treatment Unit - Brazilian Nuclear Industries UTM/INB, as well as to receive the treated effluent from this nuclear facility, coming from acid drainage from waste piles of Osamu Utsumi uranium mine (or Pit Mine Lake Osamu Utsumi). Downstream from the Antas reservoir, located Bortolan reservoir, near of the Poços de Caldas city (Minas Gerais state), part of the sub-basin of the Antas River as AR. There are no reports of studies considering the operating characteristics, ecology and diversity of the protozooplankton and bacterioplankton communities to the Bortolan reservoir, and trials conducted to date the Ferrari [3] and Rodgher et al. [4].

The three bodies of water above (Pit Mine Lake, Antas and Bortolan reservoirs) is an odd water system to be studied in Brazil and worldwide. Since the effluent comprises in nature (PML), this effluent after treatment and release into the environment (AR, P41 point) and downstream of the release point of the effluent; in other words, the Bortolan reservoir (BR).

The conducting of the a limnology study these water bodies contribute decisively to the rationale and expansion of ecological study these systems, currently managing continental aquatic systems cannot ignore the basic limnological advanced knowledge to promote effective management and long term Brazilian aquatic systems [5]. Therefore, it is imperative for licensing bodies and inspection of nuclear facilities to generate knowledge about the Sub-Basin of the Antas River, in order to support decision-making regarding the treatment and management of radioactive wastes and effluents. In this context, the present study was to contribute to the knowledge of aquatic ecosystems cited, since we lack information about the qualitative and quantitative characterization of important components of the microbial food web (planktonic protozoa and bacterioplankton), as well as the characterization abiotic factors in these environments. From seasonal and spatial study, this study was conducted to assess the possible influence of effluents from UTM/INB on the microbial food web along with aspects of the limnology of Antas and Bortolan reservoirs.

1.2 Objectives

- Perform qualitative and quantitative assessment of the protozooplanktonic community and quantitative bacterioplanktonic community in water samples from Antas and Bortolan reservoirs, as well as the Pit Mine Lake Osamu Utsumi;

- Determine the inter-relationships of physical and chemical variables with the seasonal and spatial distribution plankton community (protozooplankton and bacterioplankton) in water samples from Antas and Bortolan reservoirs, as well as the Pit Mine Lake Osamu Utsumi;

- Adapt and deploy protocol of the independent technical cultivation Hybridization Fluorescence "*In Situ*" (FISH) in water samples from the Pit Mine Lake Osamu Utsumi and Antas and Bortolan reservoirs for detection of microorganisms belonging to Archaea domain (extremophiles).

2. METODOLOGY

2.1 Experimental Design

Water samples coming from PM (Pit Mine Lake - PM), Cab (upstream discharged point), P41 (point receive the effluents), P14S (point downstream at the P41) points; all in the Antas reservoir and, BR-S point (point localized in the middle of Bortolan reservoir - BR). The campaigns were collected in the October/08, January/09, April/ 09 and July/09 months for the physical and chemical analysis and the microbial food web (protozooplankton and bacterioplankton) analyses.

2.2 Physical and Chemical Measurements

The following chemical and physical measurements were performed: pH and temperature (pH selective electrode combined with temperature sensor: WTW-320), dissolved oxygen were made with the aid of a model WTW Oximeter and electrical conductivity (conductivity brand Digimed). For the determination of the concentration of suspended solids total, organic and inorganic used the gravimetric method described in Teixeira; Tundisi; Kutner [6]. The chlorophyll concentrations were determined according to the methodology described in Marker et al. [7]. For the determination of nutrient concentrations nitrite and silicate was used the technique described by Golterman, Clymo and Ohnstad [8], nitrate and total nitrogen to the methodology described by Mackereth; Heron; Tailing [9]; ammonia to the method described by Koroleff [10]; total phosphorus to the technique described by Valderrama [11] and dissolved total phosphate and inorganic phosphate method described in Strickland; Parsons [12].

The hardness analyze were performed by atomic absorption spectrometry (Varian - Model Liberty RL Sequential ICP-OES). The fluoride concentration was estimated by potentiometric method based on the methodology described in Nascimento et al. [13]. The sulfate (UV-Visible spectrophotometry) were done in the ASTM [14]. Uranium and thorium analyzes (UV-Visible spectrophotometry with arsenazo III) were performed as in Fukuma et al. [15]. The determination of total metals manganese and zinc (Mn and Zn) was performed according to the methodology described in APHA [16] and determination of concentrations were performed in an atomic absorption spectrophotometer.

The values of chemical variables of water samples from the AR and RB reservoirs were compared with the limits established by CONAMA Resolution 357/05 [17] and Normative Deliberation Joint COPAM / CERH MG - n. 1 [18]. Amounts of thorium and uranium in water samples from the Antas reservoir (P41 and P14S) were compared to the limits set by Letter No. 50/SLC June 1997 the Nuclear Energy Commission [19].

2.3 Microbiological measurements

In the months of Oct/08, Jan/09, Apr/09 and Jul/09 identifications and quantifications were done for protozooplankton, bacterioplankton and independent technical cultivation Fluorescence Hybridization "*In Situ*". Water samples coming from PM, Cab, P41, P14S, and BR-S points were collected, fixed and identified according to: Edmondson [20]; Foissner; Berger [21], Lee et al. [22] Minchin [23]; Mikrjukov; Patterson [24], Page [25]; Pennak [26]; Regali-Seleghim et al. [27].

For the estimation of chlorophyll biomass, chlorophyll values were transformed into biomass carbon using the conversion factor of 67 according to Eaton et al. [28].

For the independent technical cultivation Fluorescence Hybridization "*In Situ*" was used a mix of probes EUB338-I, EUB338-II and EUB338-III to identify the major domain Bacteria and the probe ARCH915 specific for the identification of large domain Archaea; beyond negative control NON338 according Daims et al. [29]. The probes were labeled with Cy3 fluorescent dye (Indocarbocyanine, HE Filter Set 20 - Zeiss, wavelength: 549 nm excitation and 562 nm emission) according Glockner et al. [30]. The methodology used was described by Amann; Krumholz ; Stahl [31], Amann [32]; Glockner et al. [33] modified by Ronqui [34].

2.4 Statistical Analyses

2.4.1 Multivariate Analysis

The Principal Component Analysis (PCA) [35] and Canonical Correspondence Analysis (CCA) was applied to compare the distribution of abiotic environmental parameters, such as the physical and chemical variables and the biotic sampling in PM , Cab, P41, P14S and RB-S points throughout the study conducted seasonally.

For the PCA computer program was used multivariate statistical package MVSP which is a program for Windows 95/98/NT 3.1. CCA was used for the correlation of protozooplankton species composition with the abiotic variables, and the significance ($p < 0.05$ or 5%) of the canonical axes verified by the Monte Carlo test [36]. The computer program was used CANOCO 4.5.

2.4.2 WILCOXON Test

The Wilcoxon test was used to compare two treatments to independent technical cultivation Fluorescence Hybridization "*In Situ*": treatment one using probes EUB338-I and ARCH915 labeling with the fluorescent dye Rhodamine Red-X and the treatment two using probes EUB338-I, EUB338-II, EUB338-III and ARCH915 labeling with the fluorescent dye Cy3. The data are obtained through the pairing scheme, and the significance ($p < 0.05$ or 5%). The

goal of the Wilcoxon test was to compare the performances of each subject (or pairs of subjects, ie, the positive findings for the analyzes using probes stained with Rhodamine Red-X and, the positive results seen with probes labeling with Cy3 dye to check if significant differences between the results in both cases [37].

3. RESULTS

3.1 Limno-chemical and microbiology variables

According to the results observed for the planktonic protozoa in water samples coming from the UTM/INB in the PM point, the lowest protozooplanktonic density was $1x10^4$ cels.L^{-1} PM point (Jul/09) and, the highest protozooplanktonic density was $3x10^4$ cels.L^{-1} in PM point (Jan/09). For protozooplanktonic density values in AR and BR points (Cab, P41, P14S and BR-S) ranged from $0.03x10^4$ cels.L^{-1} (Cab, July/09) to $25x10^4$ cels.L^{-1} (BR-S April/09).

The lowest density of bacterioplankton was $0.06x10^9$ cels.L^{-1} (Apr/09) observed in water samples coming from UTM/INB in PM point evaluated in this study and, the highest bacteria density was $0.37x10^9$ cels.L^{-1} (Jan/09) in the PM. For density values of bacterioplankton in AR and BR points (Cab, P41, P14S and BR-S) values ranged from $0.05\ x10^9$ cels.L^{-1} (P14S, Apr/09) to $1.0x10^9$ cels.L^{-1} (BR-S jul/09).

According to the results obtained for planktonic protozoa observed in water samples coming from UTM/INB in PM point, the lowest biomass was protozooplankton $158.8897x10^2$ µg C.L^{-1} (Oct/08) and the highest biomass of protozoa in the same point, PM was $284.6325x10^2$ µg C.L^{-1} (Apr/09). For protozooplankton biomass values in the points Cab, P41, P14S and BR-S ranged from $0.0049x10^2$ µg C.L^{-1} (Cab, July/09) to $43.1688x10^2$ µg C.L^{-1} (BR-S Apr/09).

The results for the estimation of phytoplankton biomass recorded in water samples coming from UTM/INB in PM point showed that the lowest amount of phytoplankton biomass was lower than the detection limit in Oct/08 and; the highest phytoplankton biomass in the PM was 7.2025 µg C $x10^2$. L^{-1} (Apr/09). For values of phytoplankton biomass in the points (Cab, P41, P14S and BR-S) values ranged from 0.2010 µg C $x10^2$. L^{-1} (Cab, Jan/09) to 16.5222 µg C $x10^2$. L^{-1} (RB-S Jul/09).

The PCA applied using the values of physical and chemical variables: total suspended materials (TSM), chlorophyll *a*, total phosphorus (total P), dissolved oxygen (DO), pH, total nitrogen (total N), electrical conductivity water, fluoride, sulfate, manganese, zinc and uranium, as well as the values of protozoa total density (DTProto), protozoa total biomass (BTProto), phytoplankton biomass (BIOFHYTO) and total bacterial density (DTBac) allowed the extraction and interpretation of two factorial axes that together explained 70.2% of the variation in results. Axis 1 explained 46.7% while axis 2 explained 23.5%. Axis 1 represented a negative relationship between the variable pH (with variables BTProto, zinc, uranium, manganese, sulfate, fluoride and conductivity), with extremely positive coordinate and variables BTProto, zinc, uranium, manganese, sulfate, fluoride and conductivity negative coordinates.

The axis 2 is formed by positive coordinates P total, TSM, DO, chlorophyll *a*, total bacteria density (DTBac) and protozoa (DTProto) and the negative coordinate total N (Figure 1).

The CCA held; extracted two canonical axes that explained a total of 55.7% of the variation in the density values of the protozooplanktonic community and this percentage 69.7% could be explained by abiotic variables considered. The correlations between the protozooplanktonic community density and abiotic factors (pH, silicate, nitrate, dissolved total phosphate, OSM, Chl *a*, TSM, inorganic phosphate, nitrite, ISM, fluoride, uranium, zinc, manganese, hardness, conductivity, sulphate, thorium, ammonia, total nitrogen and dissolved oxygen) were greater than 0.9. Both the first canonical axis (F ratio = 4.244, p = 0.002) and all others were significant (F ratio = 2.231, p = 0.002) (Table 1). Table 1 eigenvalues and correlations, as well as representative diagram canonical axes 1 and 2 taken from the protozooplanktonic density values are presented below (Figure 2).

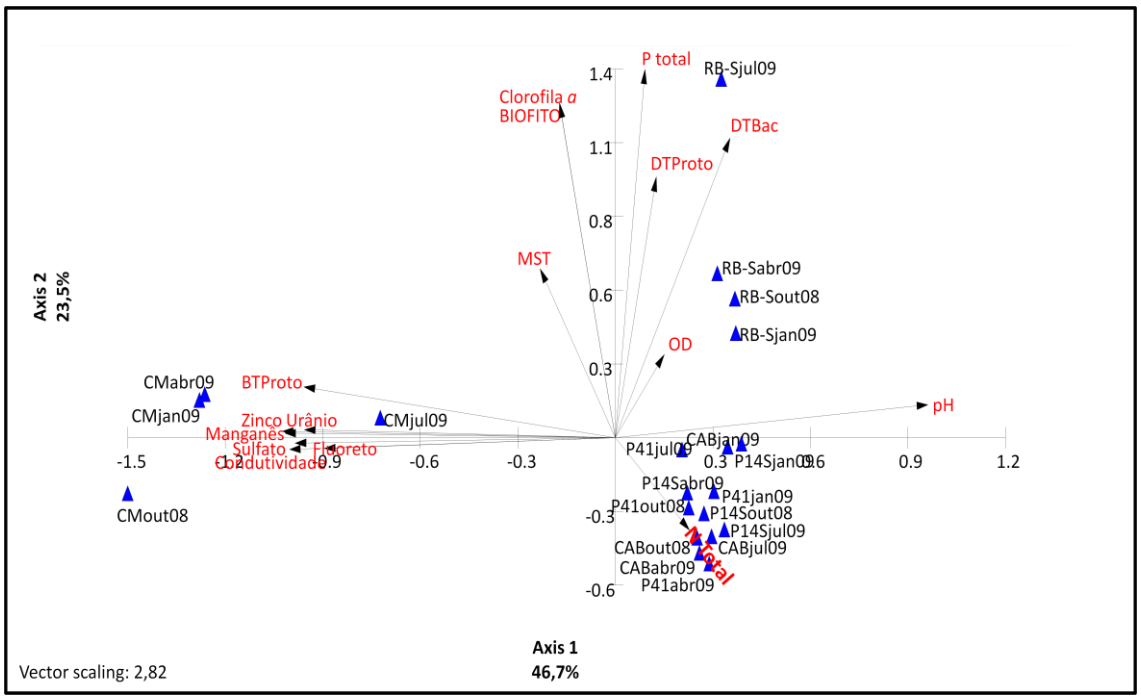

Figure 1: Diagram representing the factorial axes 1 and 2 extracted from the Principal Component Analysis applied to the results of surface.

Table 1: Results of the Canonical Correspondence Analysis and test of significance of the canonical axes (Monte Carlo test).

Axes	1	2
Species-environment correlations	0.999	0.903
Cumulative percentage variance of species data (%)	37.7	55.7
Cumulative percentage of species-environment relation (%)	47.3	69.7
Summary of Monte Carlo test		
Test of significance of first canonical axis: eigenvalue = 0.704		
F-ratio = 4.244		
P-value = 0.002		
Test of significance of all canonical axes : Trace = 1.489		
F-ratio = 2.231		
P-value = 0.002		

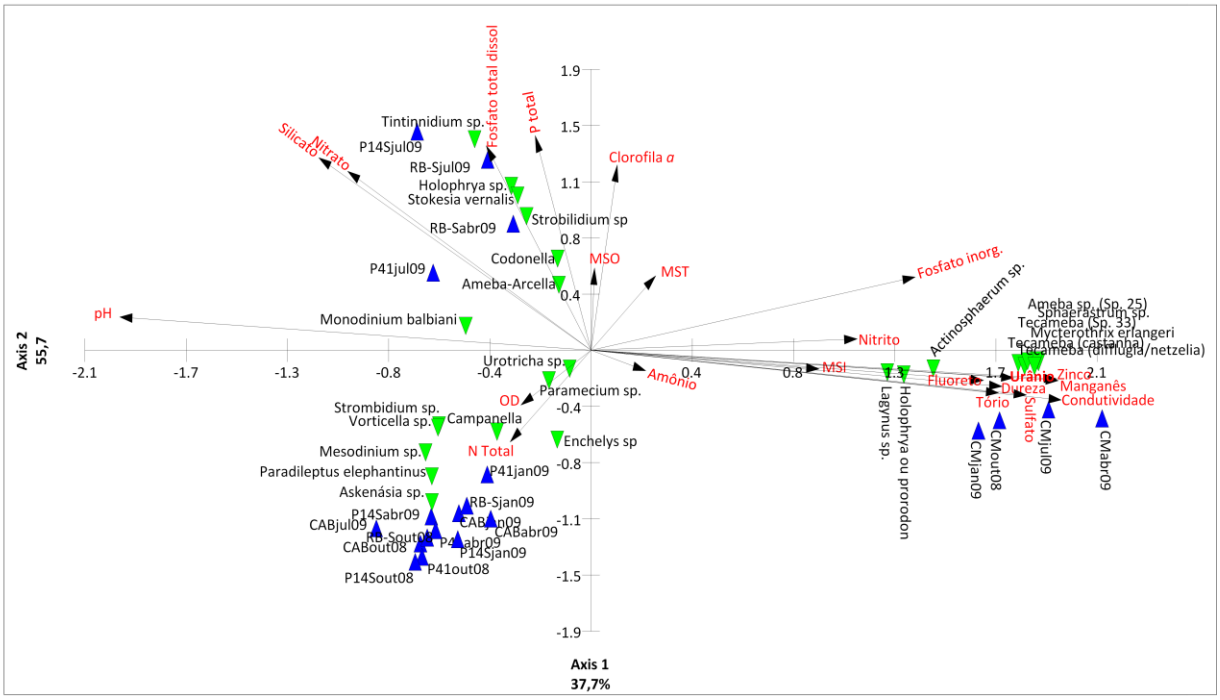

Figure 2: Diagram of the CCA ordination points in Surface (PM, Cab, P41, P14S and RB-S) in October 2008, January, April and July 2009.

3.2 Fluorescence Hybridization "*In Situ*"

In Figures 3a and 3b below, one can observe the results obtained for the samples stained with DAPI (blue cells) as well as fluorescent hybridization "*in situ*" with the probe ARCH915 labeled with the fluorescent dye Cy3 (red blood cells). The figures are coming from water samples UTM/INB at point PM, collected on April 17, 2012.

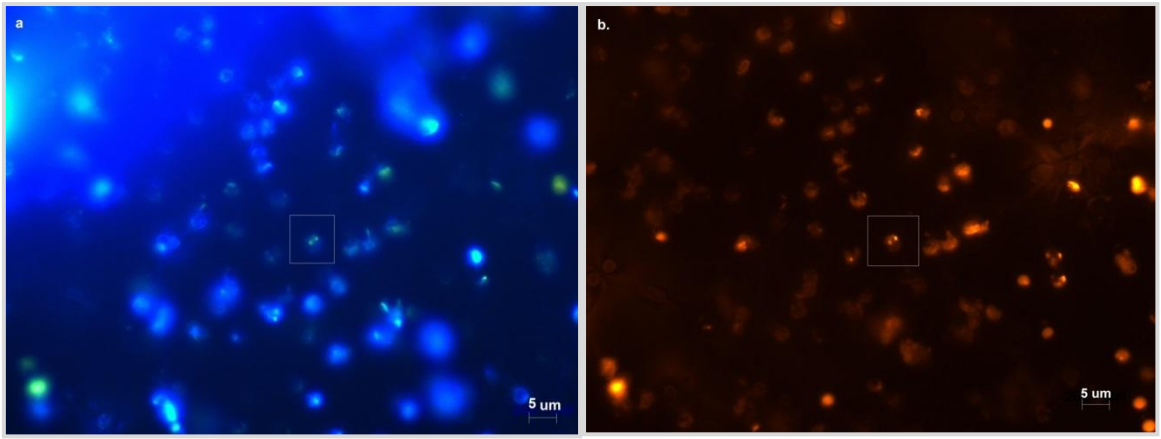

Figure (3a): Cells stained with DAPI and (3b) hybridization with ARCH915 probe, Cy3 dye.

In the figures above it can be seen the results obtained with the probes labeled with Cy3 fluorescent dye together with the suitability of the method described by Amann; Krumholz; Stahl [31]; Amann [32]; Glockner et al. [33] and modified by Ronqui [34] to the environments in this study.

Figure 4 shows the number of microscope fields evaluated in epifluorescence microscopy, consisting total cells stained with the fluorescent dye Dapi, as well as the number of microscope fields was observed where hybridization of bacterial cells across the different probes used. For the adaptation of the methodology were two test steps, where the first was performed using probes EUB338-I and ARCH915 labeling with the fluorescent dye Rhodamine Red-X and in the second test step a mix to Bacteria domain with the probes: EUB338-I, EUB338-II, EUB338-III and other probe to ARCH915 domain, both labeling with Cy3fluorescent dye. The latter being the best results observed.

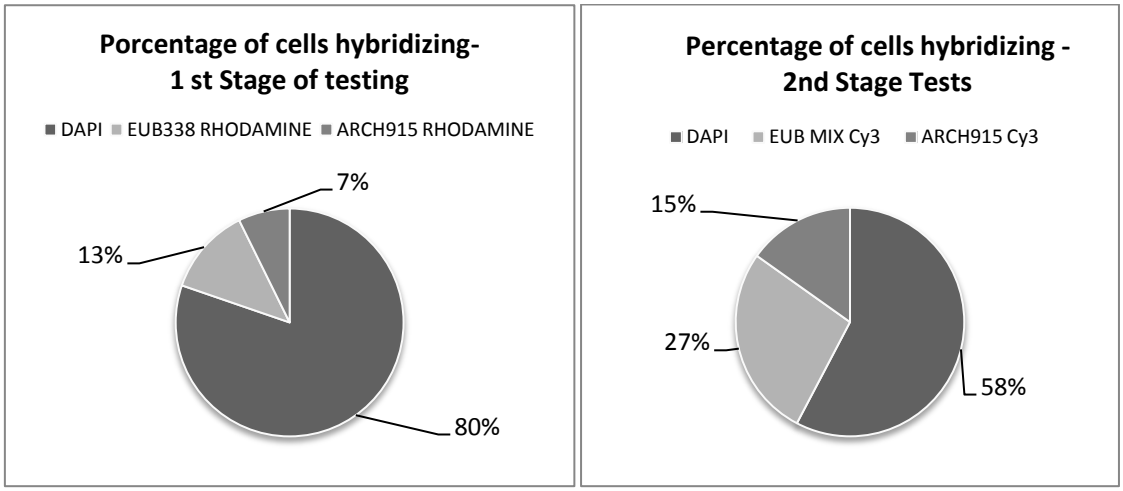

Figure 4: Comparison between the percentage of positive cells (hybridized) in 1st Step Test and 2nd Test Step forward the total percentage of cells visualized (DAPI).

Table 2 shows the results for the statistical analyzes, the significant difference between the positive results obtained from the use of probes labeled with the dye Rhodamine Red-X and

the positive results obtained with the FISH technique improvement after the use of probes labeled with the fluorescent dye Cy3.

Table 2: Results Wilcoxon test for the comparison between the results for the positive and ARCH915 EUB338 probes labeled with fluorescent dye Rhodamine Red-X and to mix the probes EUB338 I-II-III and ARCH915 labeled with the fluorescent dye Cy3.

Wilcoxon Test				
	Valid	**T**	**Z**	**p-value**
Rhodamine X Cy3	12	9.500000	2.314170	0.020659
p Value				< 0.05

Thus, according to the results, we observed significant developments in the understanding of the FISH technique, as well as the results throughout this study, in order, deploy and standardize the independent technical cultivation Hybridization Fluorescent "*In Situ*" (FISH) in a acid mine lake as Pit Mine Osamu Utsumi and the Antas and Bortolan reservoirs.

4. DICUSSION

4.1 Limno-chemicals and microbiology analyses

The Pit Mine Lake Osamu Utsumi together with the points of the Antas (Cab, P41 and P14S) and Bortolan (BR-S) reservoirs of showed significant differences in the physical, chemical and protozooplankton and bacterioplankton communities well how to estimate phytoplankton biomass, confirmed by principal component analysis (PCA) and canonical correspondence analysis (CCA).

According to the PCA could distinguish clearly the three water bodies in this study, where the acid mine lake (PM) was characterized by an environment of extreme physical and chemical characteristics, presenting positive relationships with high values of chemical variables and the lowest pH values, and positive relationship with higher protozooplankton biomass.

Moreover, AR characterized as an intermediary between the environment PM and the BR, being related to the values of both nutrients (for example, chlorophyll and total N), to micro-organisms evaluated, for pH values near the acidity as well as chemical variables during this study.

When assessed values for the chemical variables AR has a worse chemical water quality when compared to BR; probably the lowest water quality in AR is related to influences of mining effluents released by UTM/INB, possibly released treated effluent inadequate.

The Bortolan reservoir proved to be the water body with better quality chemistry among the three water bodies assessed and closely related nutrients concentrations, chlorophyll *a*, the high density values of the protozooplankton, bacterioplankton and phytoplankton biomass, as

well as at higher pH values. The BR showed totally negative correlated about chemical variables evaluated.

According to Ferrari [3], the highest concentrations of nutrients and chlorophyll *a* at Bortolan reservoir there are associated with low electric conductivity values, hardness, sulfate and fluoride, explain the higher zooplankton density and therefore the best water quality in this environment, when compared to UTM/INB in PM point and the Antas reservoir.

In general, it was found that the Antas reservoir receives greater interference from mining UTM/INB being impacted by inorganic effluents. Already Bortolan reservoir for being under the influence of the urban Poços de Caldas city, suffer greater influence of effluents from domestic and industrial surroundings.

Regarding the results obtained for the CCA analysis front abiotic variables and density values of protozooplankton community was possible to detect a pattern of gender distribution of planktonic protozoa over the water collection points evaluated in this study. It was observed that some chemical properties of water exerted a positive effect and / or no detrimental effect on the protozooplanktonic community structure recorded in acid mine lake (PM), showing organisms tolerant to such environmental conditions. According Ledin; Pedersen [38] that evaluated the environmental impacts of mining tailings and the role of micro-organisms present in these wastes, the authors inferred that the occurrence of eukaryotic organisms with high abundance, such as *Amoeba* sp. could act as efficient predators of bacteria present in the leachate, ie on iron oxidizing bacteria. Moreover, Kamjunke et al. [39] who evaluated the vertical differences in the plankton composition of an extremely acidic mine lake recorded the presence of heliozoa as *Actinophrys* sun as the top predator in these lakes from acid mine and a few ciliates and rizopodes (amoebas). This information corroborates the genera of protozoa identified in PM point.

Kihlman; Kauppila [40] also mentioned in their study that the ecological effects of metals on the microbiota in acidic mine lakes can be suppressed in mining environments due to adaptation of local aquatic biota to withstand naturally high levels of these metals.

Strong relationship was observed between some genera of protozoa (*Tintinnidium* sp., *Holophrya* sp., *Stokesia vernalis, Strobilidium* sp., *Codonella cratera*, amoeba *Arcella* sp. and *Monodinium balbiani*) and amounts of nutrients and organic matter to the points P14S Jul/09, P41 Jul/09, BR-S Jul/09 and BR-S Apr/09 (dry and cold season). According to Finlay; Esteban [41], the grazing exercised by protozoa on micro-organisms, mainly on bacterioplankton in the water column appear to stimulate the entire microbial community, possibly by increasing the turnover rate of essential nutrients present in the water column, which otherwise would remain trapped in bacterial biomass. Thus, the grazing performed by protozoa stimulate the rate of decomposition of organic matter in the water bodies.

4.1 Fluorescence Hybridization "*In Situ*"

In this study the results showed that the protocols described in the literature for hybridization of bacterioplanktonic cells in acidic mine drainage, with special attention to the coming of uranium mining, as well as oligo-mesotrophic environments still had mismatches and / or gaps to be fulfilled for general use in samples of freshwater tropical regions.

In this study there was an increase in the percentage of hybridization in bacterial cells using the culture-independent method FISH methodological modifications performed after the second stage of testing. We obtained a total of 27% positive for the mix of probes EUB338 I-II-III and 15% positive for the probe ARCH915 (both labeled with Cy3 dye) the total cells stained with the dye DAPI, ie almost double hybridizations when compared to the results obtained in the first stage of testing (EUB3385 and ARCH915 labeled with Rhodamine Red-X dye). Thus, there was a significant difference for the number of hybridizations obtained in the second stage of testing, when compared to results obtained in the first step, confirming the improvement of the technique and the results compared to standardized methodology and implemented in this study.

This study has focused on performing a first scan of the water bodies assessed to confirm the presence of bacteria belonging to the domain Archaea, as well as the efficiency of the universal probe for the domain Bacteria for such environments. We tested several steps in the present method, other steps have been introduced and / or modified in order to obtain microscopic fields where it is apparent effective hybridization of bacterial cells contained in the samples of the three environments evaluated in this study.

Due to confirm the presence of bacterial cells belonging to Archaea domain, which according to the literature [42,43] could belong to *Ferroplasmas*, *Termoplasmales* and *Sulfolobales* bacteria and extreme environments typically encountered in mining and source of acid pH. Effort sample for further study should be conducted both in acid mine lake Osamu Utsumi as the reservoirs under its influence, ie Antas and Bortolan reservoirs aiming identify archaea inhabit such environments and subsequently aid in the understanding of the biochemical processes that occur there, providing information for possible remedial action.

Thus, the deployment and standardization of the FISH technique in the present study was an attempt to unite the numerous knowledge generated over two decades of applicability and optimization of the FISH technique by numerous researchers that could help in solving the problems and constraints found for using the FISH technique to assess the bacterioplankton community in water bodies with extreme characteristics. It is noteworthy that, the need to develop specific methodologies for each type of environment or ecosystem chosen for study is needed in order to better applicability of the technique forward differences in physical and chemical characteristics of water bodies, differences between bacterial gifts communities, cell contents, growth rate or other artifacts.

5. CONCLUSIONS

The Antas reservoir was considered an environment with water quality intermediate between UTM/INB and Bortolan reservoir. In this water body (AR) chemical conditions in the months of Oct/08 and July/09 were related to the impact of mining, which probably released the treated effluent in improper conditions;

Regarding the values of density and biomass protozooplanktonic community at Bortolan reservoir showed higher values when compared to the AR and PM point, probably due to better chemical water quality and higher nutrient concentrations recorded in this water body;

In general, it was found that the Antas reservoir undergoes a greater influence the mining UTM/INB being impacted by compounds of inorganic origin. Already Bortolan reservoir for being under the influence of the urban Poços de Caldas city, suffer the greatest impact of compounds of organic origin;

Regarding the results obtained after the fitness methodology independent cultivation Hybridization Fluorescent "In Situ" in the acid mine lake Osamu Utsumi and Antas and Bortolan reservoirs; confirmed the presence of bacteria belonging to the group of extremophiles, or whether the archaeobacteria for the three bodies of water in this study;

It was concluded that the independent cultivation technique (FISH) is applicable to oligotrophic water bodies with low pH values and with high water concentrations of dissolved chemicals but, in order to maximize its effectiveness is necessary to adjust several methodological steps and; continuous optimization of protocols is of paramount importance.

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