ADRIANA M. TORRES BALLESTEROS

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do título de Doutor em Ciências.

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DECLARAÇÃO

Em adendo ao Certificado de Isenção **CEP-ICB nº 410/2010**, de 10/08/2010, e por solicitação da Profa. Dra. *Vivian Helena Pellizari*, do Instituto Oceanográfico, informo que o título do projeto de doutorado da aluna *Adriana Maria Torres Ballesteros* foi alterado para *"Impacto de hidroelétricas brasileiras nas mudanças climáticas: Micro-organismos associados à emissão de metano em reservatórios"* sem modificações de seu conteúdo, conforme sugerido pela banca de qualificação.

São Paulo, 25 de maio de 2016.

Prof. Paulo Zanotto, Ph.D.
LEMB-ICB-USP

Prof. Dr. Paulo M. A. Zanotto
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Prof. Dr. Anderson de Sá Nunes
Coordenador da CEUA-ICB/USP

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“ It is good too have an end journey toward; but is the journey that matters, in the end. ”

Ernest Hemingway

ABSTRACT

Torres-Ballesteros AM. Impact of Brazilian hydropower on climate change: microorganisms associated with methane emissions in reservoirs [Ph. D. thesis (Microbiology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

Brazil generates most of their electricity from Hydropower, however carbon footprint energy from this source has been questioned in the last years. One of the main critics is that methane emission is still not included in assessments of environmental impact. We studied the microbial ecology related with this greenhouse gas, measuring abundance of methanogenic and methanotrophic microorganisms in hydropower reservoirs. We found a strong correlation between relative abundance of these microbial groups and methane fluxes in the air–water interface. Was demonstrated that relations in gene abundances correspond with methane cycle in the six ecosystems studied. As reported in previous works, we confirmed that geographical location influences methane emissions and microbiology related to carbon fixation in aquatic environments. According to multivariate analysis, oxygen, geophysical and climatological parameters shape the balance of microbial communities related with methane cycle. Also we collected samples of sediment in São Marcos River, area chosen for a new hydroelectric power plant, and tested the methanogenic potential incubating the sample under anoxic gas different carbon source conditions. Methane production was measured during three months and analysed by gas chromatography. After incubations were completed, an aliquot was taken for nucleic acid. Our results showed a significant difference in methane production rates and lag times among the microcosms. The earlier production of methane from acetate microcosm over time confirms the importance of this process in this environment. Methanol is a substrate used exclusively by methanogenics, and thus, might explain the shortest time for the production of methane in the cultures with this carbon source. The potential methane emission in the sample was confirmed with the increasing of gas production during incubation and the amount of methane produced depends of carbon source type. These conditions also have a significant effect in the community structure of methanogenic Archaea. Findings from this work would provide scientific evidence to mitigate methane emission and manage hydroelectric development in tropical countries, one of the central sources of energy. Information acquired in this work also will contribute to achieve the challenge for future hydropower projects: settles the reduction of emissions greenhouse gases and guarantee the supply of energy demands.

Keywords: Microbial ecology. Climate change**.** Renewal energy. Carbon cycle. Greenhouse gases. Methanogenesis. Methane oxidation.

RESUMO

Torres-Ballesteros AM. Impacto de hidrelétricas brasileiras nas mudanças climáticas: micro-organismos associados à emissão de metano em reservatórios [tese (Doutorado em Microbiologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

Brasil gera a maior parte de sua eletricidade a partir de hidrelétricas, no entanto energia a partir desta fonte tem sido questionada nos últimos anos pela pegada de carbono. Uma das principais críticas é que a emissão de metano que ainda não está incluído nas avaliações de impacto ambiental. Nós estudamos a ecologia microbiana relacionadas com este gás de efeito estufa, medindo abundância de bactérias metanogênicas e metanotróficas em reservatórios hidrelétricos. Descobrimos uma forte correlação entre a abundância relativa destes grupos microbianos e fluxos de metano na interface ar-água. Foi demonstrado que as relações em abundâncias de genes correspondem com a regulação de metano nos seis ecossistemas estudados. Também confirmamos que a localização geográfica influencia as emissões de metano e a dinâmica de micro-organismos relacionadas com a fixação de carbono em ambientes aquáticos. De acordo com a análise multivariada, oxigênio, parâmetros geofísicos e climatológicas moldam o equilíbrio das comunidades microbianas relacionadas com o ciclo de metano. Também foram coletadas amostras de sedimentos no rio São Marcos, área escolhida para uma nova usina hidrelétrica, e testamos o potencial metanogênico incubando a amostra sob anóxia e em diferentes condições da fonte de carbono. A produção de metano foi medida durante três meses. Após as incubações, uma alíquota foi feita para o extração de ácidos nucleicos. Nossos resultados mostraram uma diferença significativa nas taxas de e tempos de produção de metano entre os microcosmos. A quantidade de metano produzido a partir de Acetato confirma a importância deste substrato neste ambiente. O metanol é um substrato utilizado exclusivamente para metanogênese, e, portanto, pode explicar o menor tempo para a produção de metano em culturas com essa fonte de carbono. O potencial de emissão de metano na amostra foi confirmada com o aumento da produção de gás durante a incubação e a quantidade de metano produzido depende do tipo de fonte de carbono. Esta condição também tem um efeito significativo na estrutura da comunidade de micro-organismos metanogênicas. Os resultados deste trabalho fornece evidências científicas para mitigar a emissão de metano e promover o desenvolvimento hidrelétrico, uma das fontes centrais de energia em países tropicais. As informações obtidas neste trabalho também contribuem para alcançar o desafio de projetos hidrelétricos futuros: redução das emissões de gases de efeito estufa e garantir o suprimento de demandas energéticas.

Palavras-chave: Ecologia microbiana. Mudanças climáticas. Energia renovável. Ciclo do carbono. Gases de efeito de estufa. Metanogênese. Oxidação de metano.

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LIST OF ABREVIATIONS, ACRONYMS AND SYMBOLS

%	percentage
°C	degree Celsius
"S	Southern Hemisphere
"W	Western Hemisphere
µl	Microliter
µM	micromolar
µm	micrometer
µg	microgram
ANNEL	The Brazilian Electricity Regulatory Agency
ANOVA	Analysis of variance
BSA	Bovine Serum albumin
cm	centimeter
CEFAP	Facilities Center for Research
CEPEL	Electric Power Research Center
CH_4	Methane
CO_2	Carbon dioxide
CT	Threshold Cycle
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
EC	Electrical Conductivity
EDTA	Ethylenediamine tetraacetic acid
E. coli	*Escherichia coli*
g/L	gram per Liter
GHD	Greenhouse Gases
h	hours
H_2	Hydrogen gas
HCl	Hydrochloric acid
IBGE	Brazilian Institute of Geography and Statistics
ICB	Institute of Biomedical Sciences
IPCC	Intergovernmental Panel of Climate Change

Km	Kilometer
km^2	square kilometer
L	Liter
LB	Luria-Bertani Medium
LECOM	Laboratório de Ecologia Microbiana (USP)
M	Molar
mcrA	methyl coenzyme-M reductase
***mcrA* DW**	*mcrA* copy number in bottom water
***mcrA* S**	*mcrA* copy number in sediment
***mcrA* SW**	*mcrA* copy number in superficial water
mm	millimeter
MME	Brazilian Ministry of Mines and Energy
Mmol	Milimol
mg	milligram
min	minutes
mL	milliliter
mM	milimolar
m^2	square meter
mm^2	square millimeter
$mgCH_4 /m^2 /day$	Methane milligram per square meter per day
mg/L	milligram per Liter
mV	millivolts
MCR	methyl-coenzyme M reductase
MW	MegaWatt
ng	nanogram
nM	nanoMolar
N_2	Nitrogen gas
NaCl	Sodium chloride
O_2	Oxygen
ORP	Oxidation-Reduction Potential
pb	base pairs
PBS	Phosphate-buffered saline
pMMO	particulate methane mono-oxygenase
PCA	Principal Component Analyses

PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
pH	logarithm of the reciprocal of hydrogen ion concentration
PhD	Doctor of Philosophy
pmoA	*particulate methane monooxygenase* alpha subunit
***pmoA* DW**	*pmoA* copy number in bottom water
pmoA S	*pmoA* copy number in sediment
***pmoA* SW**	*pmoA* copy number in superficial water
qPCR	quantitative Polymerase Chain Reaction
RDP	Ribossome Database Project
RNA	Ribonucleic acid
rRNA 16S	Ribonucleic acid ribossomal subunit 16_S
rRNA	ribossomal RNA
s	seconds
S/m	siemens per meter
T	Temperature
Tris	Tris-(hydroxymethyl)-aminomethane
TDS	Total Dissolved Solids
UFPA	Universidade Federal do Pará
USP	Universidade de São Paulo

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CHAPTER 1 - MICROORGANIMS RELATED TO METHANE CYCLE IN HYDROPOWER RESERVOIRS

1.1 INTRODUCTION

Research and development in energy efficiency could help in adaptation and mitigation strategies of climate change while enhancing the prospect of achieving sustainable growth. Climate change affects the function and operation energy infrastructure (as hydroelectricity) due to increasing of water demand in the coming decades, primarily because of population growth (Bates et al., 2008).

Hydropower is one of the main energy sources in tropical areas. In Brazil, 67% of the power demand is supplied by hydroelectric plants (The Brazilian Electricity Regulatory Agency, ANEEL, 2016). This renewal energy has the potential to mitigate climate change, but carbon footprint of hydropower reservoirs has been questioned in the last years (Fearnside, Pueyo, 2012; Fearnside, 2015; Hu, Cheng, 2013). Although methane (CH_4) is known as one of main greenhouse gases (GHD), the emission of this gas is still not included in assessments of environmental impact of hydroelectricity (Li, Lu, 2012). In this regard, recent works have tried to define and quantify methane emission factors in hydropower reservoirs (Barros et al., 2011; Maeck et al., 2013; Roland et al., 2010).

Brazil also developed a project (Balcar study) to obtain data related to greenhouse gas emissions from hydropower reservoirs. Besides monitoring, this information is used to estimate potential emissions (by modeling) and to establish future management practices in hydropower plants. The project was coordinated by the Electric Power Research Center (CEPEL) and headed by The Brazilian Electricity Regulatory Agency (in Portuguese, Agência Nacional de Energia Elétrica, ANEEL) with the Ministry of Mines and Energy (MME). Research teams associated to Balcar study, including Sao Paulo University (USP) and Federal University of Pará (UFPA), collected data from reservoirs located in different biomes. Professor Artur da Costa da Silva from UFPA leads the microbiology group, in which this PhD work is framed.

In freshwater ecosystems, organic carbon sources (carbohydrates, long-chain fatty acids and alcohols) are mineralized and a proportion of final products are channeled in to CH_4 biogenesis (Liu, Whitman, 2008); by anaerobic microorganisms called methanogens (Conrad, 2009). However biological processes related with methane formation (methanogenesis) remains unexplored in reservoirs sediments, mainly in tropical areas, where carbon cycle is more productive (Raddatz et al., 2007) and methane emissions seem to be higher than temperate areas (Yang et al., 2014a).

Methanogens diversity and metabolic capabilities are usually associated with substrate chemistry (Conrad, 2007); as well as other microbial communities respond to composition, quantity, and variety of carbon molecules in their environment (Hernández, Hobbie, 2010; Wang et al., 2013). Therefore, organic carbon input management could be a key element in reducing methane emissions from reservoirs (Bergier et al., 2014); to date no study has been performed to verify the effect of carbon in methanogen communities and CH_4 fluxes in hydropower reservoirs.

Carbon input differences in reservoirs can modify methane formation at community and molecular level, resulting in changes of methane emission to the atmosphere (Jugnia et al., 2005). Also metabolic capabilities of methanogens could be different in response to organic matter changes. After flooding soil during hydropower reservoir construction, carbon and oxygen varies considerably (Friedl, Wüest, 2002) and could influence methanogenic communities. To test this hypothesis was designed an experiment with sediment samples from São Marcos River, area chosen for a new hydroelectric power plant. Chapter Two present results of methanogenic potential from those samples incubated in microcosms simulating flooded, anoxic and different carbon source conditions. Additionally, data from microcosm in those conditions can be an indicator of land use changes effects in microbial communities.

Methanogenesis is very well described in freshwater sediments (Borrel et al., 2012), and recently it has also been detected in the water column (Bogard et al., 2014). Bacterial communities (methanotrophs) consume a proportion of methane produced by methanogens (methane oxidation). The final methane flux result from the balance between those two microbial processes linked to a variety of physical factors, as geometry and hydrodynamic of reservoirs (Bambace et al., 2007; Ometto et al., 2013) (see figure 1).

Analyses described in Chapter Three shows how methane fluxes in the air–water interface relates with abundance of methanogens and methanotrophs. Also, from data collected *in situ* was possible to infer which parameters shape the balance of microbial communities associated with methane cycle in reservoirs. Chapter Three presents relative abundance of methanogenic and methanotrophic communities in six Brazilian hydropower projects: Xingó, Balbina, Tucuruí, Três Marias, Segredo and Funil. Findings from this work would provide scientific evidence to mitigate future methane emissions and manage hydroelectric development in tropical countries.

Among innumerous methods to study microbial communities from sediments and soils, incubation in microcosm has the great advantage to enrich organism involved in specific metabolism (as methanogenesis or methane oxidation) (Konopka et al., 2015). Microbial diversity response to environmental changes could be monitored with new generation sequencing of 16S rRNA gene (methodology employed in Chapter Two). Besides 16S rRNA gene, Nunoura et al., (2008) suggested the use of functional genes as alternative molecular marker for methanogens and methanotrophic communities in environmental samples (without enrichments or incubations). In Chapter Three quantitative Polymerase Chain Reaction (qPCR) was used to detect those functional genes associated with methane cycle; since it is rapid, sensitive and a high efficiency technique (Colwell et al., 2008; Nunoura et al., 2008; Sharkey et al., 2004; Yu et al., 2005).

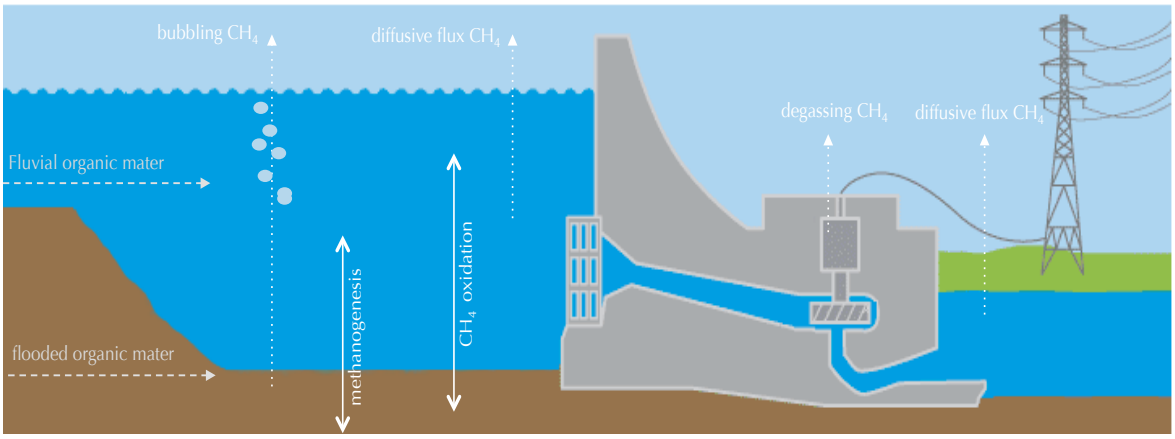

Figure 1 - Methane cycle in hydropower reservoirs.

Fluvial and flooded organic mater (soil, plant material, wood) is degraded to simplest compounds that are substrates for methanogenesis. Methane formation occurs mainly in sediments. Bacteria in the superficial sediment and column water oxidize a proportion of methane. The other part of methane is emitted to the atmosphere by bubbling, diffusive flux and in a degassing process at the dam. (Adapted from concepts in Bogard et al., 2014; Borrel et al., 2011; Goldenfum, 2010)

1.2 OBJECTIVES

The objectives of this study were:

(i) To measure the potential methane formation in an area designed for a hydropower reservoir and to evaluate changes in the diversity of Archaea and Bacteria communities with different carbon source conditions in sediment samples.

(ii) To estimate the abundance of methanogens and methanotrophic microorganism in six hydroelectric reservoirs using phylogenetic markers; and to identify relations between microbial communities and methane fluxes in the atmosphere-water interface.

1.3 DOCTORAL PROJECT MOTIVATION

A proportion of research related to climate change is driven by the need to mitigate effects of rising global temperatures. Existing information of greenhouse gases emissions from hydropower is not enough and there are difficulties in making decisions in energy infrastructure investment. The information generated from this project would provide scientific evidence to take actions to mitigate methane emission and stimulate hydroelectric development in tropical regions (one of the main sources of energy).

This research also will bring benefits to the scientific community interested in energy production and climate change. The results may provide some keys to design strategies in which renewable resources are used and emissions of greenhouse gases are minimized. Microbial ecology techniques will be used to test hypothesis; studying microorganisms in these environments is an innovative contribution in this field.

CHAPTER 2 - METHANE PRODUCTION IN HYDROELECTRIC RESERVOIRS: POTENTIAL METHANOGENIC ACTIVITY IN A BRAZILIAN RIVER

2.1 INTRODUCTION

Hydropower is an attractive energy source for Countries as Brazil (Caetano de Souza, 2008). Compared to other renewable electricity sources (wind and sun), hydropower supply is cheaper and can provide energy at big scale with fewer disruptions (Delucchi, Jacobson, 2011; Evans et al., 2009). Like all kinds of energy, implementation of these projects is associated with social and environmental impacts (Kaunda et al., 2012). As mentioned in the introductory chapter, the main criticism is based on the lack of GHD assessment, such as methane that had been detected in some reservoirs (Diem et al., 2012; Goldenfum, 2012; Li, Lu, 2012; Yang et al., 2014).

There are two ways of increasing GHD emissions by inserting hydroelectric reservoirs, one indirect and other direct. The first is related to the change in land use and the second is associated with the final product derived from organic matter decomposition (Bergier et al., 2014). Area flooding (forest, soils, rivers and lakes) for hydropower reservoir construction (land use change) modifies the biochemical parameters, which influence the GHD dynamics in the new environment (Abril et al., 2005). According to Fearnside (2002) a conversion area for reservoirs installation has shown increase of indirect methane emissions in the Amazon. As well as changes in the use of soil in these regions shows changes in taxonomic and functional diversity of microorganisms (Borneman, Triplett, 1997; Paula et al., 2014; Rodrigues et al., 2013), the main regulators of the methane cycle (Offre et al., 2013).

Terrestrial habitats flooding influence factors and organisms involved in the carbon cycling, and enhance production of GHD as methane. Study dynamics of those organisms (in CH_4 sources and sinks environment) is essential to develop more sustainable strategies for energy generation from hydropower (Jugnia et al., 2005). Methanogens are microorganisms affiliated with the Euryarchaeota phylum; mediate biological methane production (methanogenesis) (Conrad, 2009). According to 16S rRNA phylogeny, methanogens are divided into seven orders: *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales*, *Methanobacteriales Methanococcales, Methanopyrales and Methanomassiliicoccales* (Borrel et al., 2013).

Methanogens become active under wet and anoxic conditions (Angel et al., 2012), and substrate availability explains, in part, the distribution of methanogenic genera. For example in freshwater lakes, the dominant and most frequently encountered methanogenic genera are: *Methanoregula, Methanosaeta, Methanolinea* (Borrel et al., 2011). Also based on substrate consumed by methanogenic archaea, they are classified in three groups: hydrogenotrophs, methylotrophs and acetotrophs. Some species of methanogens cannot be placed in one single nutritional category because share particular characteristics. To form methane hydrogenotrophs oxidizes H_2 and reduces CO_2 or oxidizes formate. Aceticlastic (or acetotrophic) methanogens utilize acetate and, Methylotrophs uses methyl compounds (as methanol, methylamines, or dimethylsulfide) to methane production (Garcia et al., 2000).

Affinity to specific methanogenic substrates opens an interesting question about the effect that would have different carbon sources after the flooding an area. This considering that metabolic resource allocation in microorganism determines ecosystem interactions and spatial dynamics (Harcombe et al., 2014). From an ecological perspective, identify dominant species and community structure response to stress (anoxic conditions and carbon source changes) constitutes an important advance test microbial adaptation assumptions (Prosser et al., 2007; Wittebolle et al., 2009). It is expected that shifts in microbial community composition following the exposure to a novel environmental regime lead to increased productivity (methane production, in the case of methanogenic archaea) and ecosystem performance (Koskella, Vos, 2015).

Microcosms were used to study sediment samples and to integrate methane production data with microbial diversity (Widder et al., 2016). Next Generation Sequencing was the technique chosen to monitoring changes after incubation experiments. Differences in carbon entry into reservoirs can modify the community structure and amount of produced methane (resulting in changes in methane emission into the atmosphere). Also metabolic pathways of organisms associated with the formation of these gases could be different, in response to changes of organic matter in flooded area. The objectives in this chapter were: *(i)* measure the potential methane formation in a Brazilian reservoir under construction and *(ii)* to evaluate changes in the diversity of Archaea and Bacteria communities with different carbon source conditions in sediment samples.

2.2 METHODOLOGY

This section describes the techniques used with samples from an area designed for a new hydropower project: Batalha. The dam is under construction; this hydropower will produce 52.5 MW of energy. According to ANNEL and Eletrobrás-Furnas, Batalha project will provide enough power for 130,000 inhabitants.

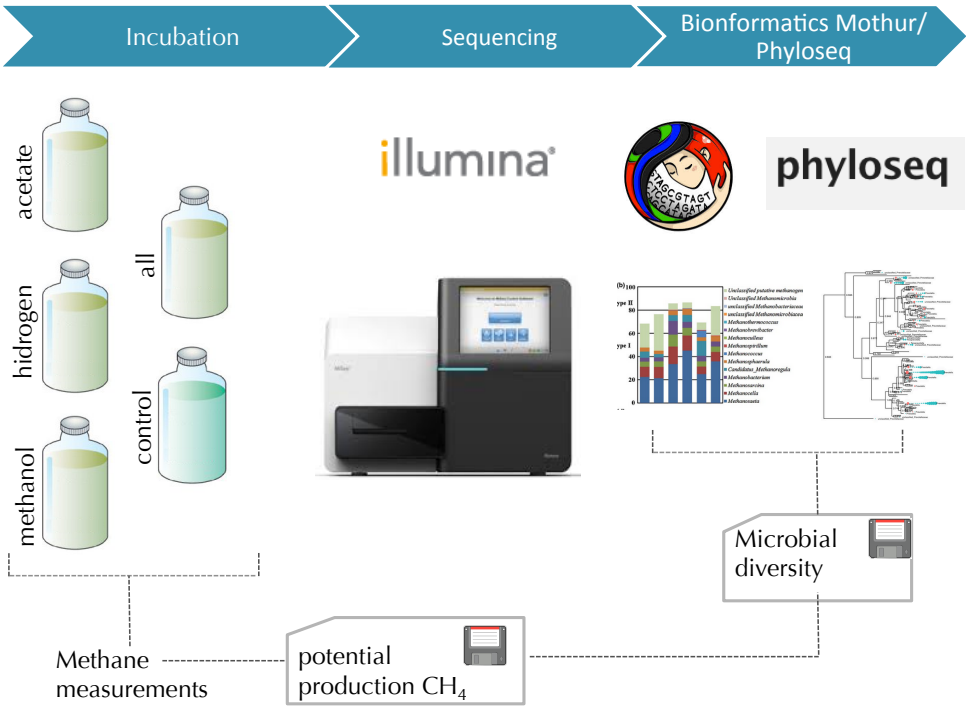

Figure 2 –Methodology for incubations in microcosm.

Figure 3 – Researchers collecting samples

2.2.1 Sampling

Sediment samples were collected near the São Marcos located between Minas Gerais and Goiás (17° 06' 04.7" S; 47° 15' 50.6" W)(area projected for a new hydroelectric power plant). The sediments were collected using a Van Veen grab. See Map in Figure 4.

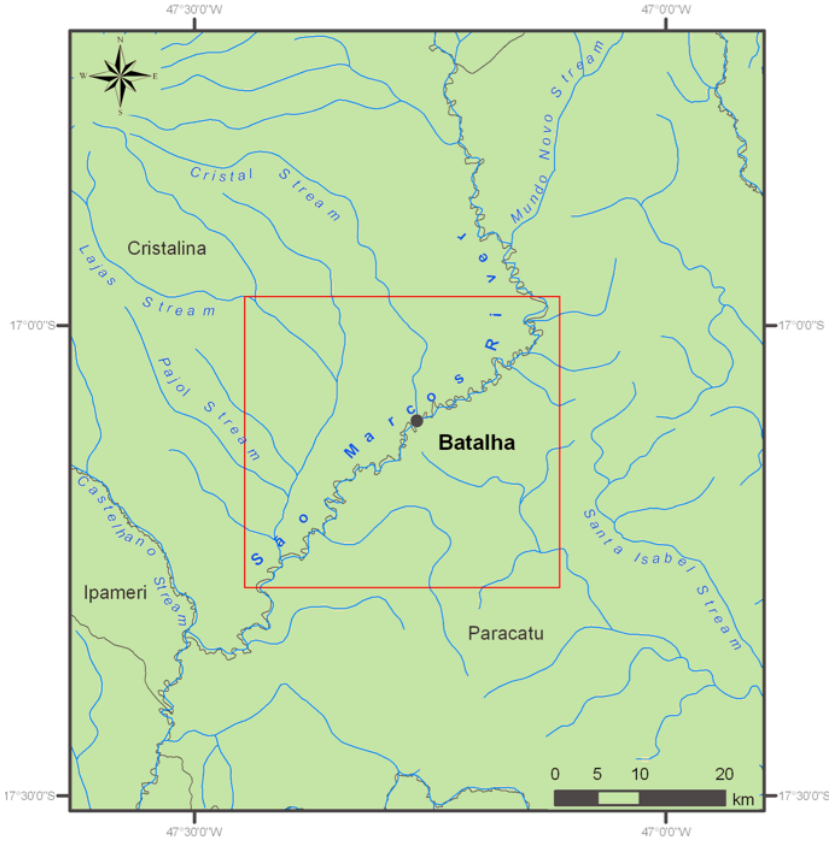

Figure 4 - Geographical location of Batalha project.

2.2.2 Incubations in microcosm and DNA extraction

Sediment samples were inoculated in microcosms containing modified Zinder medium, 60-ml glass serum bottles capped with Teflon-lined rubber stoppers and sealed with aluminium crimps (Saia et al., 2010), each with a different source of carbon (see Figure 2) with concentrations suggested by Souto et al., (2010). Thereafter, the incubation flasks were evacuated for 15 min and backfilled with N_2 three times. Microcosm containing methanol and acetate (concentration) as a carbon source were filled with a gas mixture of N_2:CO_2 (70:30). To evaluate the hidrogenotrófic metabolism, the bottles were filled with H_2:CO_2 (80:20) CO_2/H_2 mixture and the mixture in the proportion of 20% of the total volume of the headspace of bottles. The microcosms were incubated at 30 °C for 50 days. The experimental design is shown in figure 2.

During the incubation, methane production was quantified, and measurements were made in a gas chromatograph with flame ionization detection and methane concentrations were determined by direct comparison of data from a standard curve of methane 99.5%. The pattern area is determined by averaging the areas of 5 consecutive injections and the values are expressed in mmol of CH_4 (Nakayama et al., 2011). Aliquots of 1 ml each of triplicate microcosms were removed at the end of incubation time for DNA extraction. These rates were maintained using RNA ® holder (Bioagency) and frozen at -20 ºC.

2.2.3 Next generation sequencing

For this work, primers 341F ('3-CCTACGGGNGGCWGCAG-5') and 785R ('3-GACTACHVGGGTATCTAATCC-5') were selected to amplify the V3 and V4 region of the 16S rRNA gene in bacteria. Then primers 519F ('3-CAGCMGCCGCGGTAA-5') and 1017R (3'-3'GGCCATGCACCWCCTCTC -5') for amplifying the same gene in archaea (Klindworth et al., 2013). *In Silico* analyses, tested by Klindworth et al., (2013), indicated cover values of 58.2% to archaea and 70.9 % to bacteria. For primers 519F and 1017R, analyses indicated 92.8% coverage values to archaea and 0% for bacteria. Samples were sequenced in Miseq Illumina platform in a system pair-end 2 x 300 pb. The sample preparation consisted of an initial amplification of the 16S rRNA gene from genomic DNA using the primers mentioned above. Each primer had specific sequence adapters for the Miseq Illumina platform. PCR reaction (Step 1) 25 μL of enzyme consisted of KAPA HiFi HotStart Ready Mix (kappa Biosystems, CA), 5 ng of DNA and each primer in 0.2 μM. The amplification program consisted of an initial denaturation 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, annealing at 55 ºC for 30 s (or 67 °C for archaea) and extension at 72 °C for 30 s and a final extension step at 72 °C for 5 min.

Due to dimers of primers after the first PCR, bands related to the amplicons were excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, USA). The amplified DNA was quantified by Qubit fluorometer 1.0 (Life Technologies, USA) with the kit Qubit ® dsDNA HS Assay Kit (Life Technologies, USA). Then, 50 ng of DNA were used to carry out the second amplification reaction (2nd step), which consisted of addition rates of the amplicon in each of the 16S rRNA gene for the assembly of libraries. The rates of addition of PCR consisted of an initial denaturation 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, and a final extension for 5 min.at 72 °C.

Those libraries (amplicons with index) were purified using magnetic beads through AMPure XP beads kit (Beckman Coulter, USA). Workup consisted of addition of 56 μL of magnetic beads in each library. The plate containing the samples was placed in a magnetic rack for 2 min, supernatant was removed and 200 μL of 80% ethanol was added to wash the DNA. The 80% ethanol was removed and 25 μL of 10 mM Tris (pH 8.5) was added to each sample to elute the purified DNA. Libraries were analysed for quality by 2100 Bioanalyzer (Agilent Technologies, USA) using DNA chip Bioanalyzer 1000 (Agilent Technologies). DNA quantity was analysed by Qubit 1.0 (Life Technologies, USA) with Qubit® kit dsDNA HS Assay Kit (Life Technologies, USA). Normalization of libraries was made for 4 nM. Then, the final step of pooling, which consisted in joining all the libraries in a single eppendorf tube to send for sequencing. The samples were sent to the CEFAP-ICB (Facilities Centre for Research, *Centro de Facilidades e Apoio à Persquisa*) of the Institute of Biomedical Sciences - University of Sao Paulo.

2.2.4 Microbial diversity analyses

The sequences obtained from Illumina Miseq were processed using bioinformatics program Mothur. Individual contigs were made from the two reads for each sequence (obtained by pair end 2 X 300 bp system). Samples were identified from the indexes that were followed by primers. Then sequences were aligned with the 16S rRNA GreenGenes database (DeSantis et al., 2006). For quality control, smaller sequences than 400 bp were discarded; and sequences with ambiguous bases and homopolymers larger than 8 bp. sequences were pre-grouped following the algorithm described (Huse et al., 2010) Chimeras (3%) were removed with chimera-Slayer command in Mothur.

Taxonomic composition of the samples was determined using Mothur, gene 16S rRNA sequences classification ARB SILVA data (Quast et al., 2013). The confidence limit for this classification was 65%. For the variety of calculations, the libraries were normalized by reducing the total number of sequences in each library to a common size for all samples (23,000). Using a 97% identity, the sequences were grouped into operational taxonomic units (OTUs). From this result we calculated the diversity indices (observed wealth, ACE, Shannon Smith-Wilson). Were used to estimate beta values as the dissimilarity index Curtis Bray, the dissimilarity index Sorenson based on abundance), and the dissimilarity measure. The *composition and community structure was based on the absence or presence of certain OTUs.*

2.3 RESULTS

2.3.1 Methane production potential

Results showed a significant difference in methane production rate at the exponential phase. Treatment with all sources of carbon and treatment with methanol, methane production began 10 days after the initial day of incubation. Microcosms with hydrogen and acetate at this stage produce nothing or less methane compared with the other treatments (see figure 5). At the end of incubation (at stationary phase), the concentration of methane in the treatment of acetate was higher compared to the other microcosms (see Figure 6).

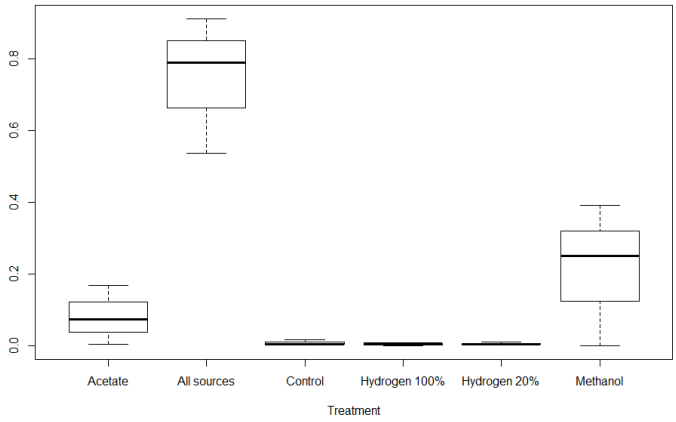

Figure 5 - Methane production potential at the exponential phase.

Amount of methane produced in the exponential phase (10 days): from acetate (Acetate), all substrates (all sources), without substrates (Control), 100% hydrogen (Hydrogen), hydrogen 20% (Hydrogen 20%) and methanol (Methanol).

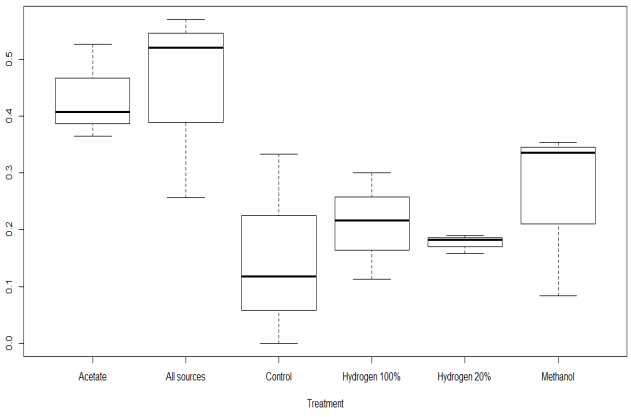

Figure 6 - Potential for methane production the stationary phase.

Amount of methane produced in the stationary phase (40 days): from acetate (Acetate), all substrates (all sources), without substrates (Control), 100% hydrogen (Hydrogen), hydrogen 20% (Hydrogen 20%) and methanol (Methanol).

2.3.2 Relative abundance of microbial communities

As the amount of methane produced depends on the carbon source type, the structure of Bacteria and Archaea communities changes in relation to the substrate and incubated after subjecting the samples to anaerobic conditions. Based on the data, the most abundant kind in environmental samples (not incubated) is *Rhodonobacter* (see Figure 7).

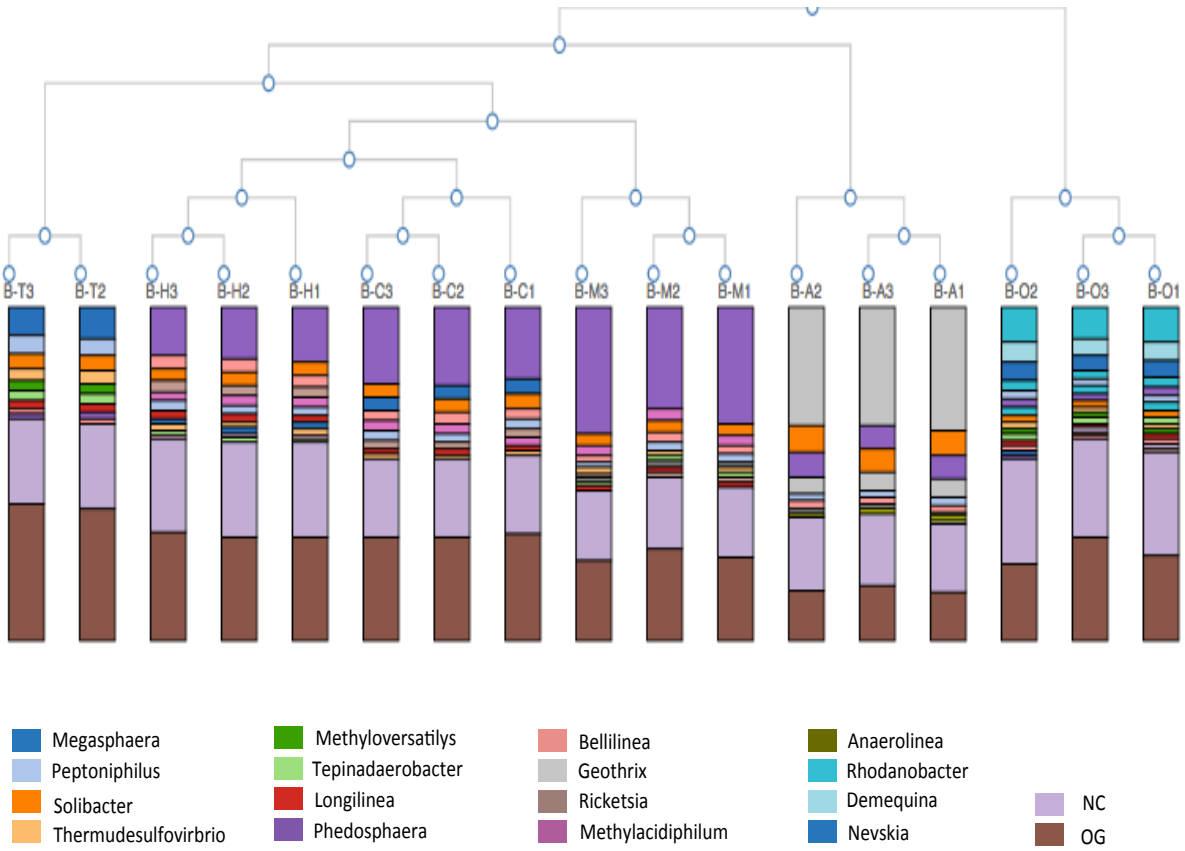

Figure 7 - Grouping of samples similarity according to the relative abundance of bacteria Domain.

The dendrogram shows a hierarchical clustering of samples based on the genus level ratings. Replicas of samples for sequences of the microcosms bacteria primer incubated with all substrates (B-T2, B-T3) with hydrogen (B-H1 B H2-B-H3), methanol (B-M1 B -M2, M3-B), with ethyl (A1-B, A2-B, A3-B). Replicas to sequences obtained from environmental DNA (B O1, B-O2, B O3) and microcosms control without substrate (B C1, B, C2, C3, B). NC: not classified sequences. OG: other genres.

A third interesting aspect of our results was that microcosm communities diverged in composition compared with the source communities. For Archaea, mean microcosm similarity was 30.3±0.7 compared with 44.7±1.8 for the initial sediment samples, whereas for Bacteria, the corresponding microcosm and sediment similarities were 18.9±0.6 and 30.6±1.9, respectively.

PERMDISP analysis (Clarke and Warwick, 2001) was used to test whether the dispersion between all microcosms was greater than that between inoculum: this was true for both Archaea and Bacteria. Thus different source communities diverged, rather than converged, upon colonization of the common microcosm environment. For Bacteria, where only time zero samples, which do not contain most of the inoculum bacterial diversity, gave resolvable fingerprints, we could not perform this analysis.

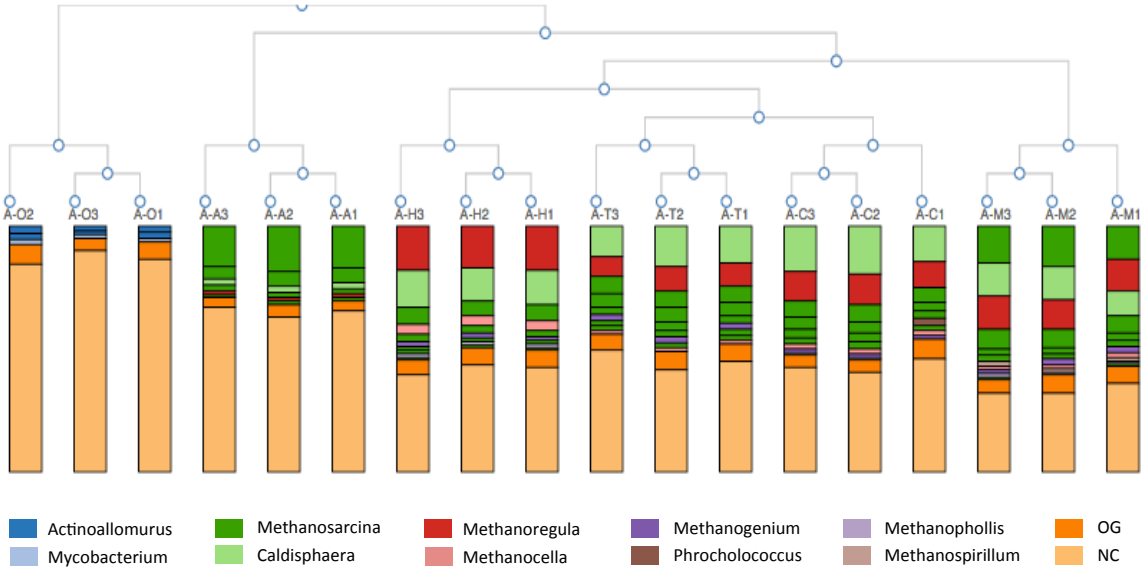

Figure 8 - Grouping of samples similarity according to the relative abundance of archaea Domain.

The dendrogram shows a hierarchical clustering of samples based on the genus level ratings. Replicas of samples for sequences of the microcosms bacteria primer incubated with all substrates (B-T2, B-T3) with hydrogen (B-H1 B H2-B-H3), methanol (B-M1 B -M2, M3-B), with ethyl (A1-B, A2-B, A3-B). Replicas to sequences obtained from environmental DNA (B O1, B-O2, B O3) and microcosms control without substrate (B C1, B, C2, C3, B). NC: not classified sequences. OG: other genus.

We next analysed the variability of the final community composition in our microcosms. As one might expect given the small size of the microcosms and the strong selection, community richness was substantially reduced compared with the inoculating sediments: mean band numbers were 22.8±1.1 for bacterial 16S rRNA genes and 14.0±0.7 for archaeal 16S rRNA genes with similar decreases for. The lower values also reflect a less even community composition in the microcosms compared with the inoculum. Bacteria also showed reduced diversity and evenness in the microcosm communities as measured by OTU number.

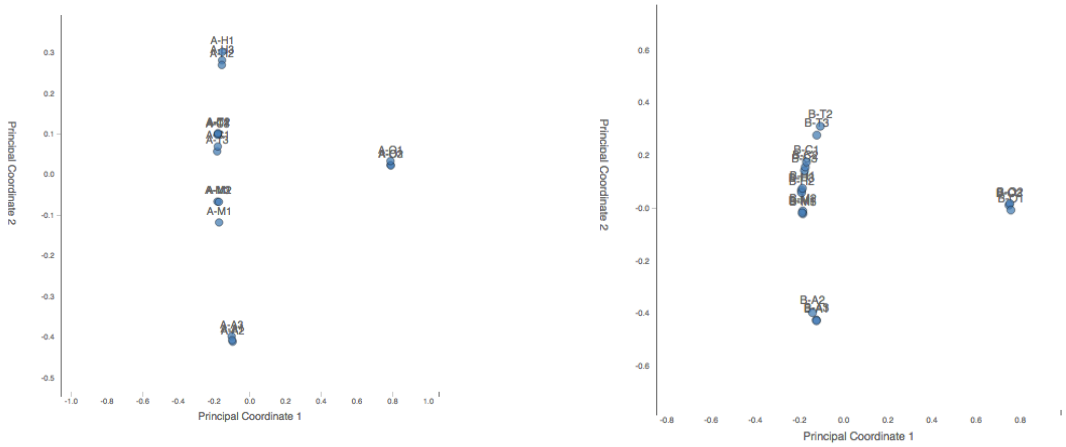

Figure 9 - Principal Coordinates Analysis (PCoA)

A) Distribution of samples based on archaeal diversity information of the microcosms incubated with all substrates (A-T2, T3-A) with hydrogen (The H1, H2-A, A-H3), methanol (A- M1, a, M2, a-M3) with ethyl acetate (a-A1, a, A2, a-A3), replicates to sequences obtained from environmental DNA (a-O1, a-O2, the O3) and microcosms of control without substrate (A C1, A C2, C3, B). B) Differences in community structure between the developed microcosms. These results show that microcosms differ in their final community compositions, whereas different source communities produce divergent microcosm communities.

2.3.3 Microbial diversity in microcosms

Table 1 - Diversity index and richness of Archaea and Bacteria in samples of all treatments.

	Archaea		Bacteria	
ID Amostra	Shannon Species Diversity	Number of Species Identified	Shannon Species Diversity	Number of Species Identified
O3	0,310	196	2,077	438
O2	0,468	236	2,216	358
O1	0,421	192	2,196	411
H3	1,274	160	1,804	322
H2	1,243	179	1,824	304
H1	1,236	188	1,793	407
T3	1,315	196	2,012	452
T2	1,446	148	1,987	358
T1	1,385	240	-	-
M3	1,509	152	1,297	308
M2	1,534	175	1,42	398
M1	1,508	240	1,368	365
A3	1,016	137	1,244	392
A2	1,124	137	1,253	294
A1	1,069	149	1,261	396
C3	1,342	114	1,526	343
C2	1,316	111	1,538	340
C1	1,461	237	1,566	475

2.4 DISCUSSION

2.4.1 Carbon source effect in the potential production of methane

In freshwater environments such as flooded lakes and soils, methane production is most commonly associated with acetate and then the hydrogen, which are the most affordable substrates in these environments (Demirel, Scherer, 2008). However, these compounds are also used by other groups of microorganisms that compete with methanogens for substrate. The methanol, in turn, is used more narrowly substrate and thus less competitive. The absence of competing microbial groups, likely explains the lowest elapsed time for methane production in the cultures fed with the methanol. However, the fact of the methanogenesis acetoclastic has resulted in a higher methane production until the end of the incubation time confirms the importance of this process in the studied environment. Also methane production with acetate addition was apparently lower than with CO_2/H_2 mixture (Figure 5 and 6) suggesting methanogenesis sediment was substrate limited, and the majority of active methanogens were rather acetoclastic than hydrogenotrophic methanogens.

2.4.2 Carbon source effect in microbial community structure

The *Methanosarcina* a genus is well distributed in various environments, this was found in all microcosms but with a lower abundance in the treatment with hydrogen. Methanosarcina organisms are metabolically versatile and can use three types of methanogenic substrate. It is the same case for *Methanoregula* and *Methanogenium* genre that were also found in all analysed incubations.

Species from *Methanospirullum* genus use compounds such as hydrogen and acetate to produce methane. In experiment it was detected only in cultures with hydrogen, as *Methanocella,* which is a strict hidrogenotrófico. *Methanofolli*s was found one of the genres that also use methanol metabolism in this microcosm he would have an advantage in the competition for substrate. They also found the sequences *Caldisphaera*; these organisms are heterotrophic and have been isolated from acidic environments and high temperatures.

According to available data and to the analysis performed in this review (Table 1), 9 known genera are mainly retrieved and represent 75% of the sequences of methanogens. The hydrogenotrophic genus *Methanoregula* (order *Methanomicrobiales*) is recovered in almost all 16S rRNA archaeal clone libraries created from freshwater lakes (Table 1) and sequences of this genus represent one-third of the 16S rRNA sequences of methanogens from freshwater lakes available in international databases (Table 1) could be noted that strains and type strains of *Methanobacterium* and *Methanosarcinaceae* were commonly cultivated from freshwater lakes by using media with high substrate concentrations. This suggests that these genera commonly occur in freshwater lakes even as rare species

In the case of domino Bacteria, *Rodonobacter* and *Nevskia* was only detected in environmental samples. The species *Nevskia ramosa* is inhabitant of the interphase air-water freshwater. The most abundant genus in microcosms incubated with acetate was *Geotrix,* wrapped in aromatic hydrocarbon degradation. The only species described like this: *Geothrix fermentans* was isolated and cultured using a medium acetate and Fe (III). The addition of acetate favoured growth of this group in microcosms under anaerobic conditions. *Pedosphaera* was a genre detected at all microcosms and environmental samples but were more abundant in the incubations with methanol, hydrogen and no substrate control (see figure 8). This genus is common in terrestrial environments but has only been described in aerobic samples.

According to the tests *in silico*, the primer pair suggested by (Klindworth et al., 2013) for Archaea was 92.8% and 0% for bacteria, although only the forward primer amplifies also for bacteria. If the amplification conditions are not ideal and the genomic DNA material contains low number of archaeal organisms, this will amplify more bacterial sequences. In environmental samples obtained were classified few sequences, For samples microcosms the sequences was sorted for arching most probably due to enrichment and incubation would increase the archaea number.

2.4.3 Changes in microbial diversity by potential land use changes

Indices calculated for bacteria show an increase in the alpha range and a decrease in beta diversity after incubations under anaerobic conditions (see in Table 1: Shannon index alpha diversity). The same pattern was observed in a study of the effect of land use change in the Amazon, where it was shown that the microbial diversity is also influenced by such environmental change (Rodrigues et al., 2013). For Archaea was observed an increase of alpha diversity in the microcosms, these changes may be related to the redundancy of functional genes; that in soils depends on the members of the microbial community (Griffith, Philippot, 2013; Levine et al., 2011;).

In regard to functional diversity, changes in land use can influence gene groups related to the carbon and nitrogen cycle (Paula et al., 2014). It is an example of diversity pmoA, a gene encoding the subunit of a mono-oxygenise, richness and fullness has been correlated with the methane oxidation rates and regulation of the flows that greenhouse gas (Levine et al. 2011). There were no sequences of organisms containing the gene, probably through the middle and substrate used in microcosms that is specific to methanogenic archaea. The organisms that oxidize methane depend on the substrate generated by methanogens; therefore a change in the use of ground could result in changes in the methane cycle. Increases in CH_4 flux in wetlands and processed soil for pasture has been attributed to changes in physical and chemical properties of the soil, such as the decrease in porosity as a result of soil compaction and increased organic matter and pH.

2.4.4 Analysis of metabolic fluxes in reservoir sediment

Methane emissions seem to be higher in tropical areas and regulation of carbon input in reservoirs is proposed to be a strategy to reduce green house emissions. Carbon input in reservoirs could influence methane production by microorganism, and consequent emission to the atmosphere. We propose to evaluate changes in methanogenic microbial communities in different levels of carbon input. In order to measure the effect of rising organic matter in flooded area, two different sediments from Brazilian reservoirs will be analyzed using stable isotope probing (SIP) and next generation sequencing with the University of Southampton and founds from the IPCC scholarship. The study will elucidate the importance of carbon input level in this system, which is key information to develop mitigation strategies for methane emission, while stimulating hydroelectric development (Annex B)

CHAPTER 3 - BALANCE OF MICROBIAL COMMUNITIES IS RELATED WITH LOW METHANE EMISSION IN HYDROPOWER RESERVOIRS

3.1 INTRODUCTION

After the international climate agreement in Paris at COP21, investors and researchers gathered at the United Nations to begin mobilizing efforts to catalyze the global clean energy transition (Davenport, 2015). In tropical countries the investment in clean energy has been directed toward the use of water resources (de Faria et al., 2015). Based in a special report from IPCC, energy from hydropower has the potential to mitigate climate change, as have less impact per unit of electricity generated when compared to other sources (Edenhofer et al., 2012). However there is an active discussion around the environmental impact of this energy source, linked to the carbon footprint of hydropower reservoirs (Fearnside, 2005, 2015; Hu and Cheng, 2013),

Direct greenhouse gases emissions of hydroelectric reservoirs dependent on the geographic location; Barros et al., (2011) found highest emission rates near the tropics when compared to other latitudes. However tropical reservoirs have been under sampled (Gudasz et al., 2010) and dynamics of microbial communities, which control methane cycle, in those ecosystems diverge from patterns in other latitudes (Sarmento, 2012).Some authors attribute these differences to temperature and carbon cycle that is more productive in tropical reservoirs (Marotta et al., 2014; Yang et al., 2014a; Yvon-Durocher et al., 2014). Reservoir age is other main variable correlated with greenhouse emissions; at initial stages (after construction) methane emissions are higher and tend to decline with time (Abril et al., 2005).

All current carbon-trading schemes to mitigate climate change are based in CO_2 but, methane emission have a larger impact on greenhouse effect (Shindell et al., 2009). Methane result from organic matter degradation in lakes and reservoirs (Tranvik et al., 2009), and two microbial process control the cycle of this gas: CH_4 production (methanogenesis) and CH_4 consumption (methane oxidation). Anaerobic microorganism (archaea) mediate methanogenesis, they use substrates obtained after mineralization of organic carbon sources (as carbohydrates, fatty acids and alcohols) to produce methane; mainly in sediments (Conrad, 2007; Liu, Whitman, 2008).

Methane could be transported by either diffusion or ebullition to the atmosphere; or can also be oxidized in water column and emitted as CO_2 (Yang et al., 2014a). Bacteria oxides methane (methanotrophs) when uses CH_4 as source of energy, and then carbon is transferred in substantial amounts to higher trophic levels (Sanseverino et al., 2012). Methanotrophic activity explains why CH_4 emissions at air–water interface are 2 orders of magnitude lower than in the mixed layer in tropical freshwater ecosystems (Morana et al., 2015).

A methane source environment implicates CH_4 generation greater than its consumption, but consider a sink when methane oxidation is greater than its production. A change in the equilibrium between methane sources and sinks can increase atmospheric emission (Wuebbles, Hayhoe, 2002). For example after a reservoir construction, atmospheric methane concentration could vary as consequence of biogeochemical cycles modifications in microbial communities dynamics.

Some studies have shown a direct link between biogeochemical processes with microbial genes (Rocca et al., 2015). *mcrA* and *pmoA* functional genes provide a suitable resolution to detect microbial communities from methane cycle (Nunoura et al., 2008). For example, Sawakuchi et al., (2015) found a strong correlation between methane oxidation and *pmoA* abundance in tropical rivers. Likewise other works with soil exhibited significant positive relationship between the abundance of *mcrA* and methanogenesis (Angel et al., 2012; Freitag et al., 2010; Watanabe et al., 2009) Gene's *mcrA* and *pmoA* codify for subunits of highly conserved enzymes that catalyzes keys steps in methanogenesis and CH_4 oxidation respectively (Luton et al., 2002; Thauer, 1998).

The *mcrA* gene codes for the alpha-subunit of the methyl-coenzyme M reductase (MCR). This enzyme catalyzes the final step in all methanogenic metabolic pathways: hydrogenotrophs, methylotrophs and acetotrophs. MCR, unique to methanogens (Thauer et al., 2008), constitutes a functional and phylogenetic marker of microorganisms involved in methane metabolism (Luton et al., 2002). Conrad et al., (2010) already used this gene to investigate the diversity, structure, distribution and ecology of methanogenic communities in freshwater lakes. The alpha subunit of a methane mono-oxygenase (pMMO), encoded by the *pmoA* gene, has also been used in environmental studies for obtaining functional and taxonomic inventories of methanotrophs (Luesken et al., 2011). Bacterial methane oxidation starts with conversion of methane to methanol and is catalyzed by pMMO.

Molecular techniques, as real time quantitative PCR, provide an improved tool for understanding how microorganism control greenhouse gas emissions in natural environment (Zhang, Fang, 2006). Include this microbial information in large-scale models constitutes a challenge to determine ecosystem functioning (Konopka et al., 2015). In hydropower reservoirs those models could be used for potential strategies to reduce greenhouse gas emissions, through managing microbial processes.

Was expected that the balance between methanogenesis and methane oxidation, quantified by *mcrA* and *pmoA* abundances, resulted in different values for methane fluxes. This Chapter shows results from analysis made in six Brazilian hydropower reservoirs (with different climate regimes). Was determined the abundance of methanogens and methanotrophs and explored the relations between microbial communities with methane flux at the water-atmosphere interface.

3.2 METHODS

This section describes the techniques used with samples from six Brazilian hydropower reservoirs: Xingó, Tucuruí, Balbina, Funil, Segredo, Três Marias. Data of methane diffusive fluxes were collected in collaboration with the National Institute of Space Research (*in Portuguese, Instituto Nacional de Pesquisas Espaciais*, INPE)

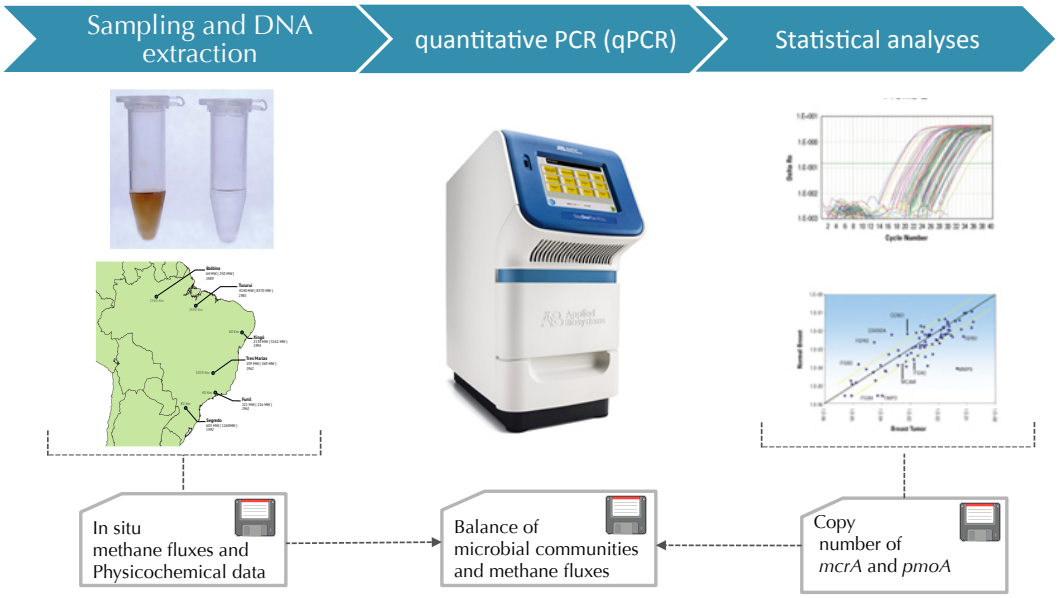

Figure 10 - Experiment scheme to detect abundance of microorganisms associated with methane cycle.

3.2.1 Methane emissions

Methane fluxes ($mgCH_4/m^2/day$) were measured using floating chambers (figure 11) with the method described by Marani and Alvalá, (2007). Additionally was used the N-20XD probe (Horiba) to determine electrical conductivity (S/m), oxide reduction potential (mV), dissolved oxygen concentration (mg/L), pH, temperature (°C) and total dissolved solids (g/L). Were made four sampling campaigns (2011 to 2012) in each reservoir (Balbina, Tucuruí, Segredo, Funil, Três Marias and Xingó) at different times of the year, see geographical location in Figure 12.

Figure 11 - Methane emissions measurements.

A). Team member getting ready to make measurements of methane fluxes. **B)** Floating chamber at the interface water - atmosphere **C)** Scheme of floating chamber method used in reservoirs.

3.2.2 Sediment and water sampling

Different stations were selected for molecular analyses in each reservoir (see figures 13, 14, 15, 16, 17 and 18). Water samples were collected using a van Dorn bottle, from two levels in the water column: one meter after surface and one meter before the bottom. Also was collected sediment using Van Veen grab.

Water samples (1L) were filtered in two stages: the first through a filter with 0.22-µm-pore size (Millipore) to remove solid particles and the second using nitrocellulose membranes with 0.22-µm-pore size (Millipore). This membranes were maintained in 5ml of a storage buffer (50 mM Tris-HCl, 500 mM NaCl, 125 mM EDTA, pH 8.0) at -20 °C prior to extraction (Graças et al., 2012). The sediment samples also were stored in this buffer at -20 °C.

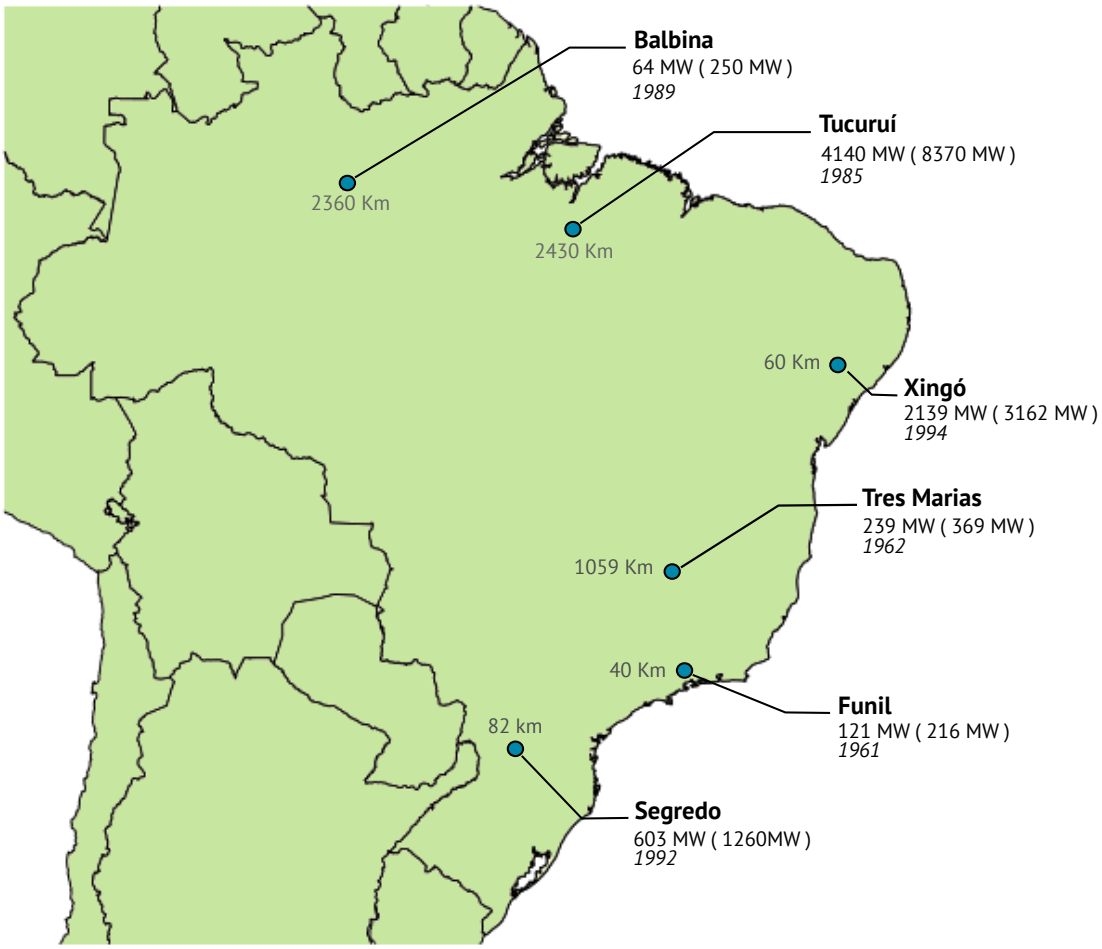

Figure 12 - Approximate geographic location of reservoirs studied.

Next to the blue dot is the number of kilometers (Km^2) flooded for the reservoir construction, down the name is the value of energy produced (MW megawatts) and between parentheses the installed power. In the last line below the reservoirs name is the starting year of operations. Figure was generated using R: A language and environment for statistical computing with library (maps) and library (mapdata).

3.2.3 DNA extraction

For DNA extraction from water filters, we used DNeasy Blood and Tissue Kit (Qiagen, USA) with the Manufacturer's protocols except for a preliminary lyse with liquid nitrogen and proteinase K (Ottesen et al., 2011). To extract the DNA from sediment was used Power Soil DNA Isolation Kit (MoBio laboratories, CA, USA) according to manufacturer's instructions. Before the qPCR reactions, DNA (from sediment and water) was purified using the OneStep-96 Inhibitor Removal PCR kit (Zymo Research, CA, USA). DNA concentrations were determined using the Qubit dsDNA HS Assay (Invitrogen, CA, USA). Finally the DNA was diluted in a proportion of 1:10.

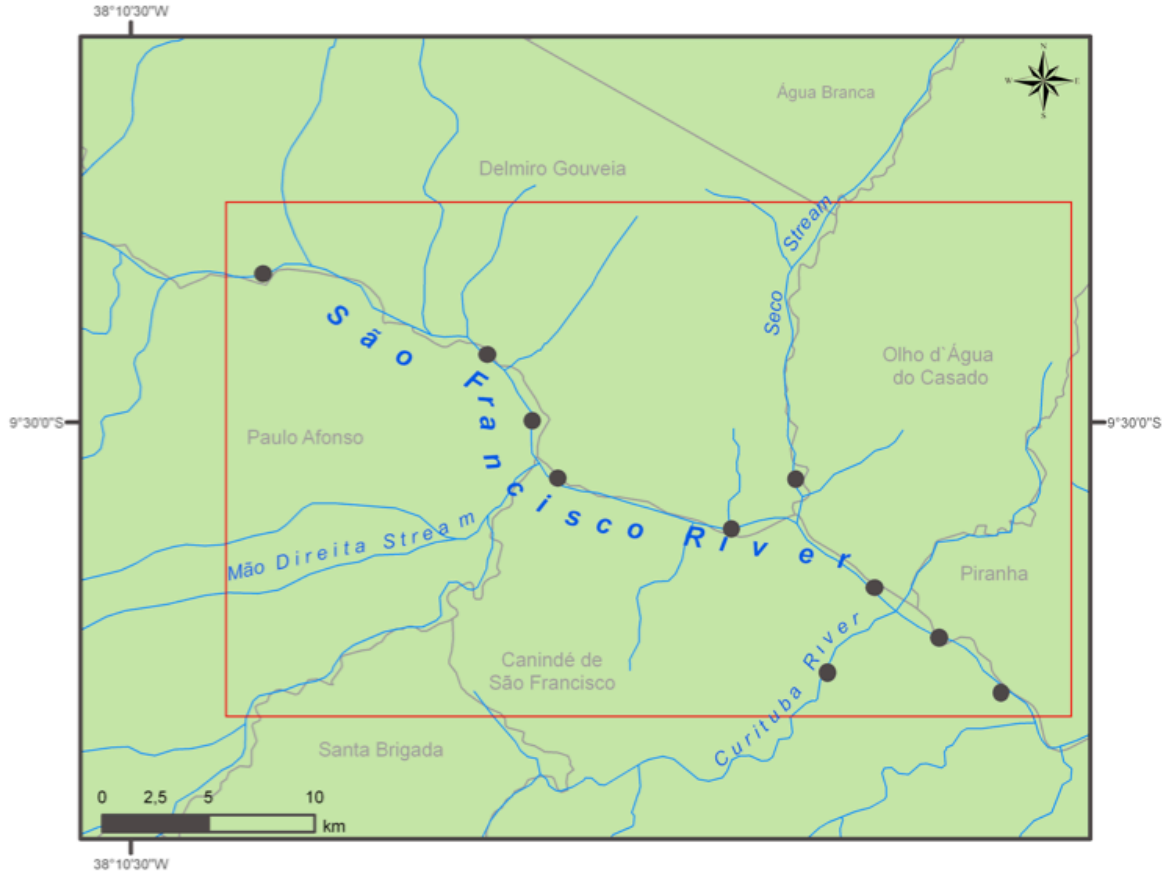

Figure 13 - Geographical location and monitored stations in Xingó reservoir.

The hydroelectric is located in the lower course of São Francisco river (the main river in the northeast region of Brazil) and covers the states of Alagoas, Sergipe and Bahia. Xingó was built for navigation, water supply and hydroelectric power generation of 3,162 MW. Dam's power station works with six Francis turbines. Reservoir area is 60 Km^2 and the length reaches about 60 km. Average temperatures in the region are around 25 ºC and total annual rainfall index is between 413-907 mm/year. The rainiest season is from May to July and the driest is between September to November. Xingó is situated in a large canyon and operates as Run-of-the-river hydroelectricity (ROR), this system characterized by a limited amount of water storage and is subject to seasonal river flows. Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics (in Portuguese, Instituto Brasileiro de Geografia e Estatística, IBGE).

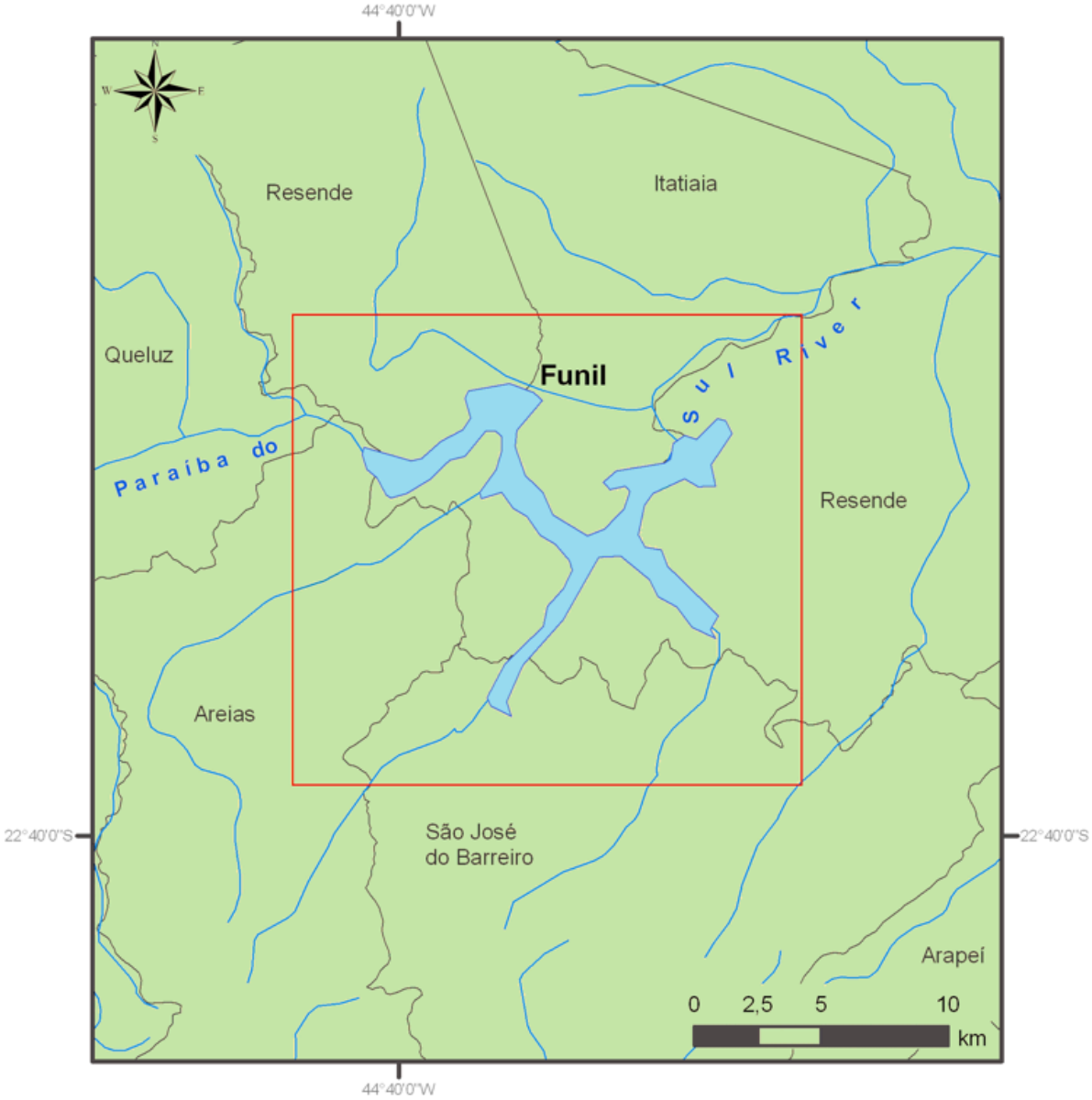

Figure 14 - Geographical location and monitored stations in Funil reservoir.

This hydroelectric Power Plant is formed in the Paraiba do Sul River, and located in the municipalities of Itatiaia and Resende (Rio de Janeiro State). The reservoir has a main channel of 35 km and two secondary channels feed by streams from Paulista basin. Funil has a flooded area of 40 Km^2 with a maximum depth of 70 m and average depth of 20 m. Hydroelectric Power Plant has a capacity of 180 MW, and works with three Kaplan turbines of 60 MW. The average annual temperature is 21 °C, in summer the average increase to 24 °C and in winter decrees to 17 ºC. The annual rainfall reaches 2500 mm, the highest is (in summer, May to September) and in winter the minimum annual rainfall is 500 mm (winter and summer were defined according to Köppen climatological classification). Despite the low installed capacity, Funil is considered and important power plant for their location (close to major consumer centers in Rio de Janeiro, São Paulo and Espiritu Santo States). Also Funil provides electricity supply to major industries, such as *Companhia Siderurgica Nacional* (CSN). Construction of the Funil dam has also reduced the frequency and intensity of floods in downstream cities (ANNEL, 2014). Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics *(in Portuguese, Instituto Brasileiro de Geografia e Estatística, IBGE)*.

Figure 15 - Geographical location and monitored stations in Segredo reservoir.

The Official name of this reservoir is Governor Ney Aminthas de Barros and is located in the Iguaçu River; between the municipalities of Mangueirinha and Reserva do Iguaçu (Paraná state). The power plant works with four generating units of 315 MW each, resulting in 1260 MW of installed capacity. The climate is temperate according to the Köppen classification; 16.8 °C is the average annual temperature and the average annual rainfall is 1897 mm. January is the hottest month of the year with an average temperature of 20.8 °C, and July is has the lowest average temperature of the whole year: 12.2 °C. Also July is the driest month with 118 mm and October is the month with the highest rainfall index, with an average of 202 mm. The hydroelectric project was executed in steps: *(i)* construction of river diversion tunnels and *(ii)* the second stage running major civil works. The plant was fully ready only after the completion of the Jordan River deviation, allowing divert part of the flow through a tunnel 4,703 m long and 9.5 m in diameter. This leads to a 10% increase in of energy produced by the plant. This was the first hydroelectric plant in Brazil to have an environmental impact project. Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics (*in Portuguese, Instituto Brasileiro de Geografia e Estatística,* IBGE).

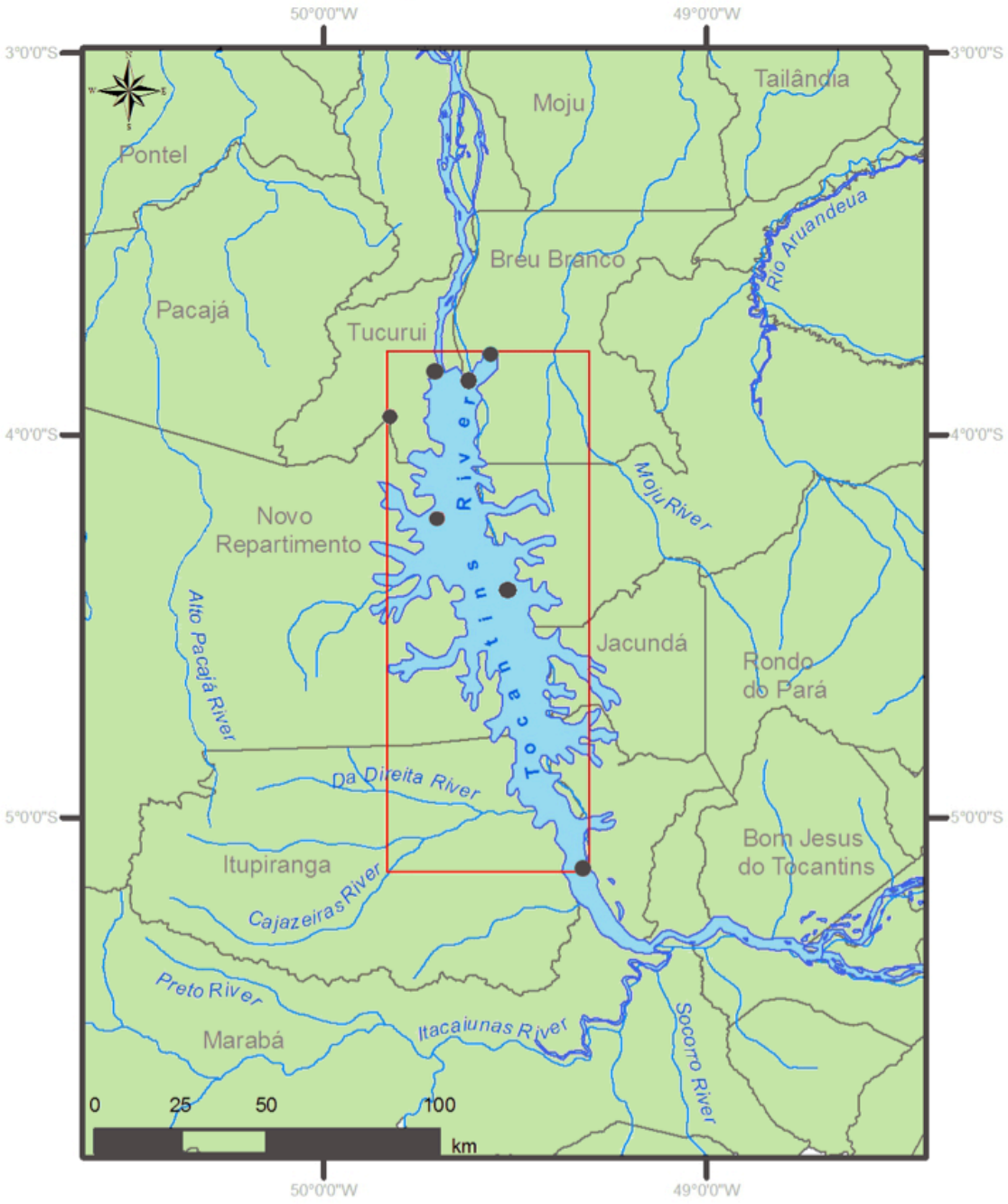

Figure 16 - Geographical location and monitored stations in Tucuruí reservoir.

The plant of Tucuruí was the he first large-scale hydroelectric project in the Brazilian Amazon rainforest, located in the Tocantins River at the municipality of Tucuruí (Pará State). The reservoir is 200 km long and has 2430 km^2 of flowed area; 88% of this area was not cleared and dead trees still remains under reservoir water. Plant works with 25 Francis-turbines and has an installed capacity of 8370 MW. Regions climate is between super-humid equatorial and equatorial humid (according to Köppen classification), relative humidity is high and oscillate from 100% to 60%. The average annual temperature is 26 ºC, with a maximum value around 32 °C and minimum of 23 ºC. Rainfall varies about 2000-2500 mm annually; from January to June precipitation is abundant, and the driest period is from July to December (ANEEL, 2014). Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics (*in Portuguese, Instituto Brasileiro de Geografia e Estatística,* IBGE).

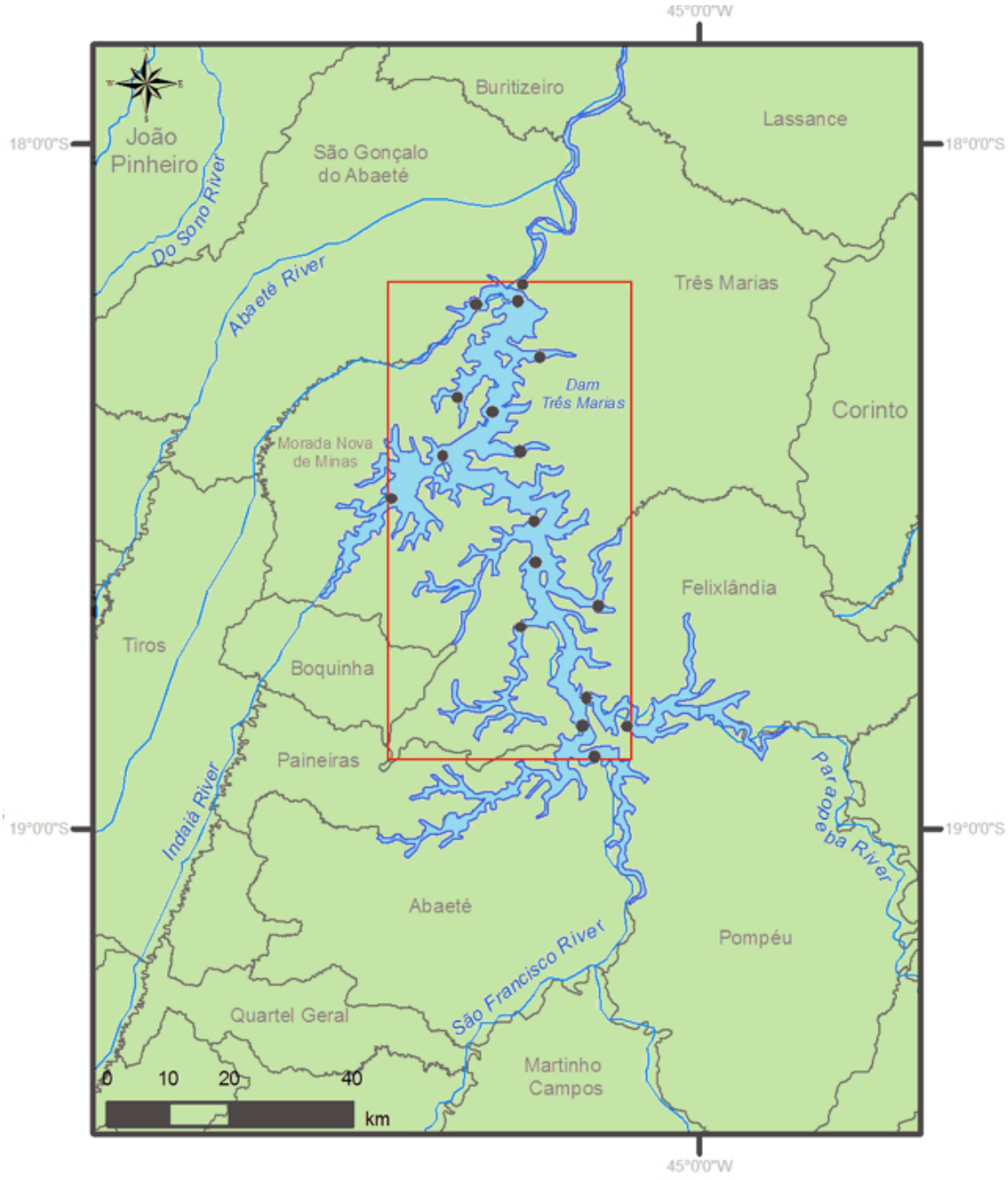

Figure 17 - Geographical location and monitored stations in Três Marias reservoir.

Três Marias dam and reservoir was built for navigation, flood control, irrigation and hydropower supply. Located in the São Francisco River (state of Minas Gerais), the dam also regulates downstream water flows to other reservoirs and power plants. Hydroelectric Power Plant has an installed capacity of 396 MW, and contains 6 turbines Kaplan. Compared to other hydropower plants, the flooded area of the reservoir (1059 km^2) is too large for the low energy produced. The region has a Tropical savanna climate (according to Köppen climate classification) and there are two seasons well defined: a driest season between April and September and a rainy season from October to March. The average annual precipitation is 1200-1300 mm and the average annual temperature is 23.5 ºC. 55 to 60% of the annual total precipitation results from the rains in November, December and January (ANNEL, 2014). Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics (*in Portuguese, Instituto Brasileiro de Geografia e Estatística*, IBGE)

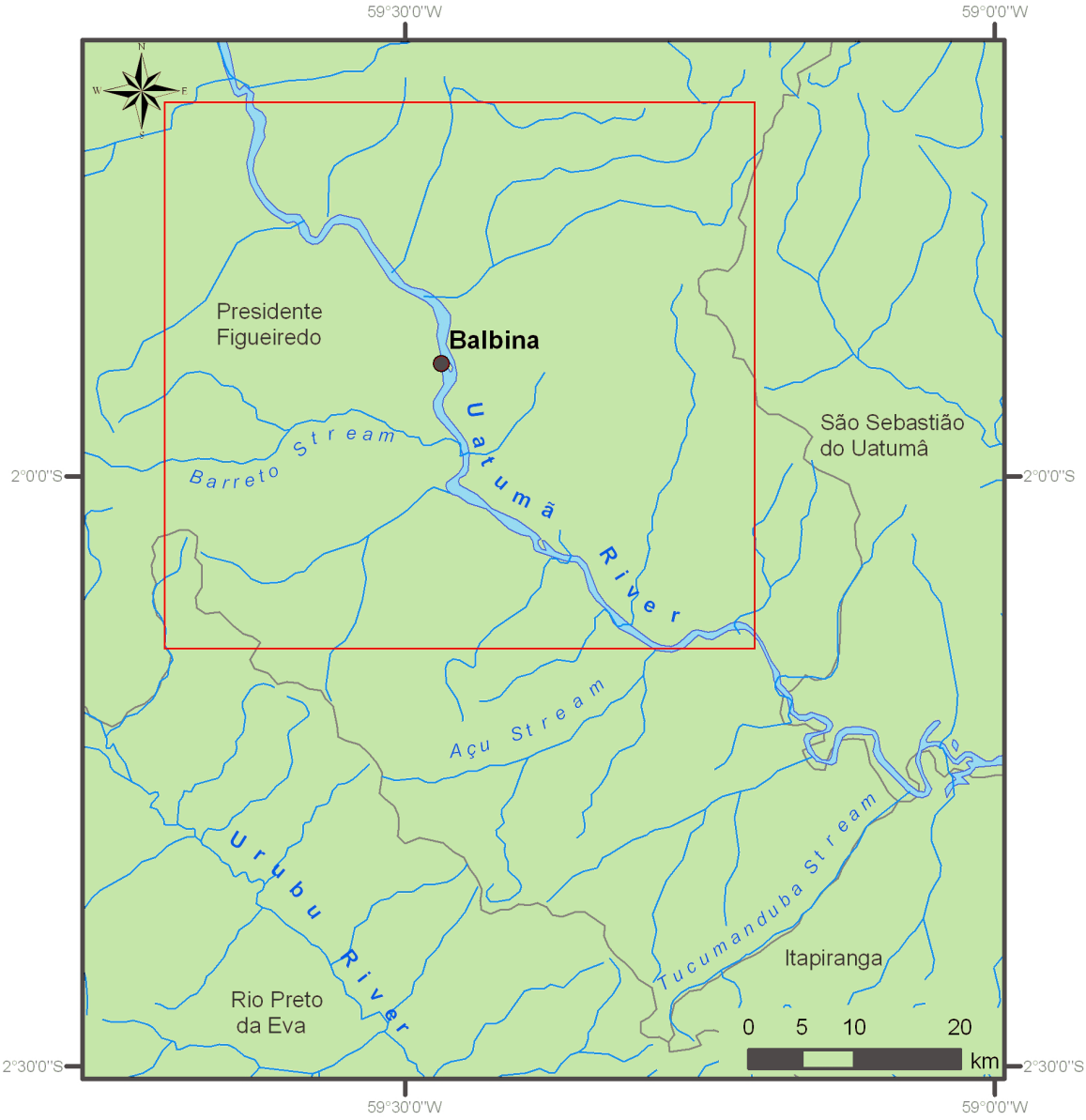

Figure 18 - Geographical location and monitored stations in Balbina reservoir.

The Balbina Dam, located in the Uatuma River (Amazonas state) has an installed capacity of 250 MW and works with 5 turbines Kaplan. The flood area is 2,360 km^2 with an average depth of 7.5 m and 210 km length. Flooded dead trees form a proportion of the reservoir; vegetation has been left to decompose occasioning water acidification and turbines corrosion. In August to November (periods of low rainfall), reservoir presents hypoxia in the hypolimnion and thermal stratification. Also shallow reservoir contains 1500 islands and bays where the water's residence is longer than the average time of over one year, increasing anoxia in the system. Balbina dam has been considered one of the biggest failures of hydroelectric projects in the Amazon. Reservoir flooded area is too large for the low energy produced (64 MW), this because the flat topography and small size of the drainage basin. According to Köppen classification climate is tropical rainforest, average annual temperature in the region is 27.1 °C and the average annual rainfall is 2975 mm. September is the hottest month of the year with an average temperature of 27.8 °C., and January has the lowest average temperature (26.6 °C) (ANEEL, 2014). Figure was generated using ArcGIS (ESRI, 2011) and a database from the Brazilian Institute of Geography and Statistics (*in Portuguese, Instituto Brasileiro de Geografia e Estatística*, IBGE).

3.2.4 Quantitative PCR

To construct the standard curve for *mcrA* and *pmoA* quantification; DNA from methanogenic (*Methanosarcina sp.*) and methanotrophic (*Methylocistis sp.*) cultures was extracted with PureLink™ Genomic DNA kit (Invitrogen, CA, USA). The *mcrA* and *pmoA* gene were amplified with primers MLAs - mcrA (Luton et al., 2002) or A189b - Cmo682 (Luesken et al., 2011) separately. PCR products were ligated into TA vector and used to transform *E. coli* (*Escherichia coli*) Top10 cells (TA cloning kit, Invitrogen) according to manufacturer's protocols. Positive clones were selected and cultured overnight at 37°C in (Luria-Bertani Medium) LB broth with ampicillin. After extracted Plasmid DNA from clones with the kit PureLink Quick Plasmid Miniprep (Invitrogen, CA, USA). Serial dilutions were made from plasmid DNA and dilutions with 10^2-10^7 copies (of each gene) were used as positive control.

Protocol for *mcrA* amplification started with a denaturation step of 10 min at 95 °C. After were made 45 cycles of 95 °C denaturation for 30 s, 56 °C to annealing for 45 s, 68 °C extension for 45 s. To allow dissociation of possible primer dimers and nonspecific amplification was carried out a final cycle of 8 s at 81 °C for fluorescence detection as described by Freitag and Prosser (2009). The final volume of the reactions was 25 µl: 5 µl DNA sample, 12.5 µl Power SYBR 2X (qPCR master mix) and forward primer (1.2 µM) and reverse (1.2 µM). Bovine Serum Albumin (BSA) (200ng, ml^{-1}) and dimethyl sulfoxide (DMSO) (5%) were added to qPCR master mix to increase the efficiency of the enzymatic reaction. The average of amplification efficiency was 97% and all amplifications were linear ($r^2 = 0.96$). Amplification of samples and standards, including negative controls, were made in triplicate. Melt curve analysis was performed after the final extension step.

For *pmoA* amplification, protocol was initiated with 10 min denaturation at 95 °C. After, were made 45 cycles: 95 °C of denaturation for 30 s, 64 °C of annealing for 45 s, 68 °C of extension for 45 s followed by a step of 16 s at 81 °C to allow dissociation of possible dimers of primers and nonspecific amplifications (Freitag et al., 2010). The final volume was 25 µl reactions: 5 µl of DNA sample, 12.5 µl of 2X SYBR Power forward primer (0.8 µM) and reverse (0.8 µM). BSA was added (200 ng ml^{-1}) the to increase the efficiency of the enzymatic reaction (Freitag and Prosser, 2010). The average amplification efficiency was 96% and all amplifications were linear ($r^2 = 0.97$). Melt curve analysis was performed after the final extension step.

3.2.5 Statistical analyses

Data of diffusive methane fluxes were expressed in mg/m^2/day, and one-way ANOVA, followed by Tukey's test, was employed to verify significant differences between measurements in reservoirs. From these data was also generated a density graph to compare distribution of the methane diffusive fluxes of in the water-atmosphere interface.

To define abundances of microbial groups was assumed that each organism has a single copy of the gene *mcrA* or p*moA*, based on sequence information of genomes, which have between one and two copies. Abundance of microbial groups was expressed as number of gene copies per ng of DNA. We transform the CT (Threshold Cycle) values for the number of copies of each gene, using the amplification curve of each plate. Data from sediment and water samples were standardized using the DNA concentration (ng/µl). The total gene abundance in each functional group was log transformed, and compared using the difference between *mcrA* copy number and p*moA* copy number. The total gene abundance in each functional group was ordered in a heat map and normalized by functional group in each reservoir, with mean and standard deviation equal to one (Paula et al., 2014). Samples were then clustered using Euclidean distances with the complete linkage method, based on their color intensity profiles (R Development Core Team 2010).

Conoco 5 and PCA method (Principal Components Analysis) was performed to identify relationship between the genes abundance and environmental variables: electrical conductivity, oxide reduction potential, dissolved oxygen, pH, temperature, total dissolved solids and methane fluxes. Simple linear regression analysis was performed to explore possible relations of CH_4 flux with the average of gene *mcrA – pmoA*.

3.3 RESULTS

3.3.1 Methane emissions

Methane fluxes in the six reservoirs (during 2011 and 2012) showed that CH_4 emissions by diffusive transport were between 0.19 and 38.4 mg/m^2/day, at water-air interface. Tucuruí, Balbina and Xingó were the hydroelectric reservoirs with the highest methane values (see figure 19). Três Marias was the hydroelectric with lower emissions values compared to the other systems, and the average of methane flow was also more homogeneous during all sampling periods (see figure 20).

Frequency distribution graph also denote that most methane measurements are around 7 mg/m^2/day, in all reservoirs. Figures 3 and 4 shows that methane emissions in Funil and Segredo systems are the most similar. Comparisons based in Turkey test only showed a significant difference in values between Três Marias and Xingó (P *Value >0,5*).

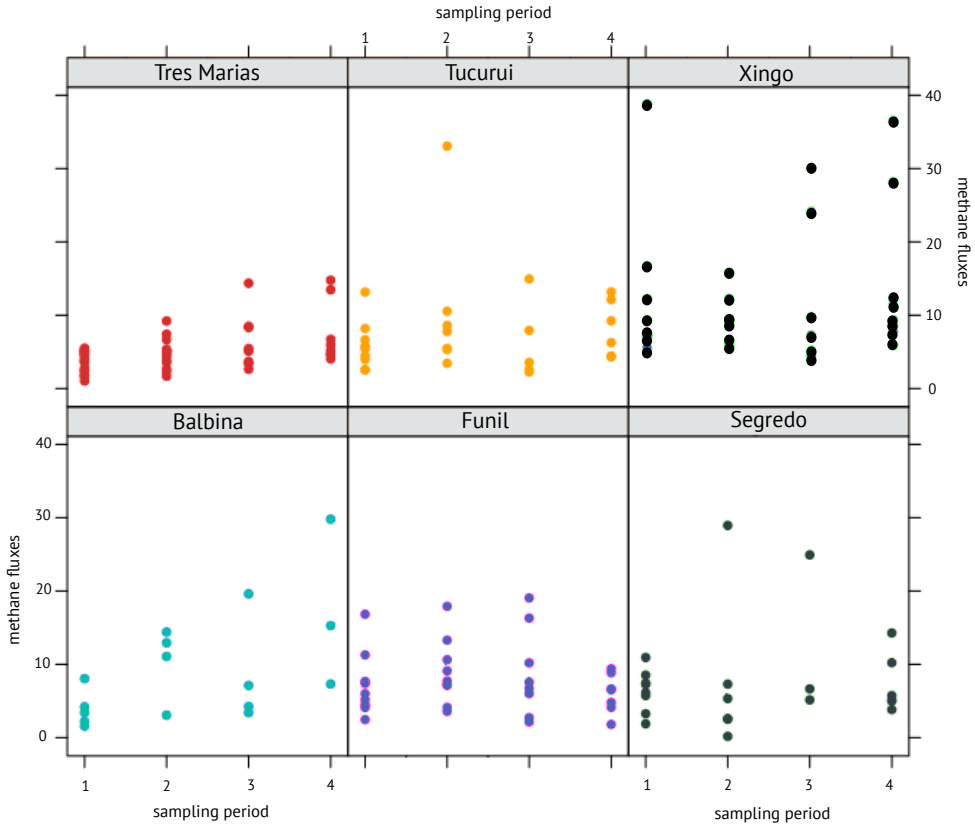

Figure 19 - Methane diffusive fluxes in the six reservoirs studied by sampling period.

Individual data points are averages from triplicate values in sampling points. Data are divided by four seasons in each reservoir (*X*-axis). Values of diffusive methane fluxes are in mg/m^2/day (*Y*-axis).

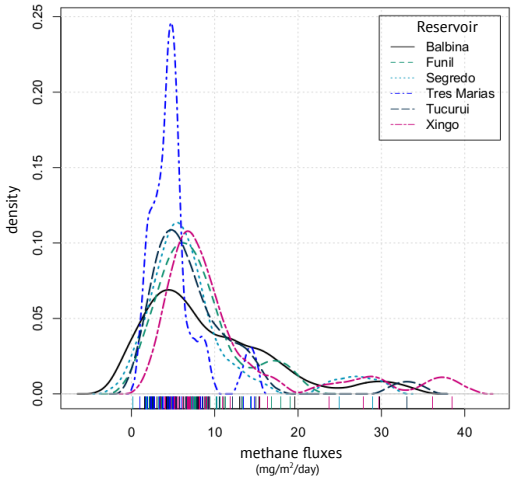

Figure 20 - Distribution of diffusive methane fluxes.

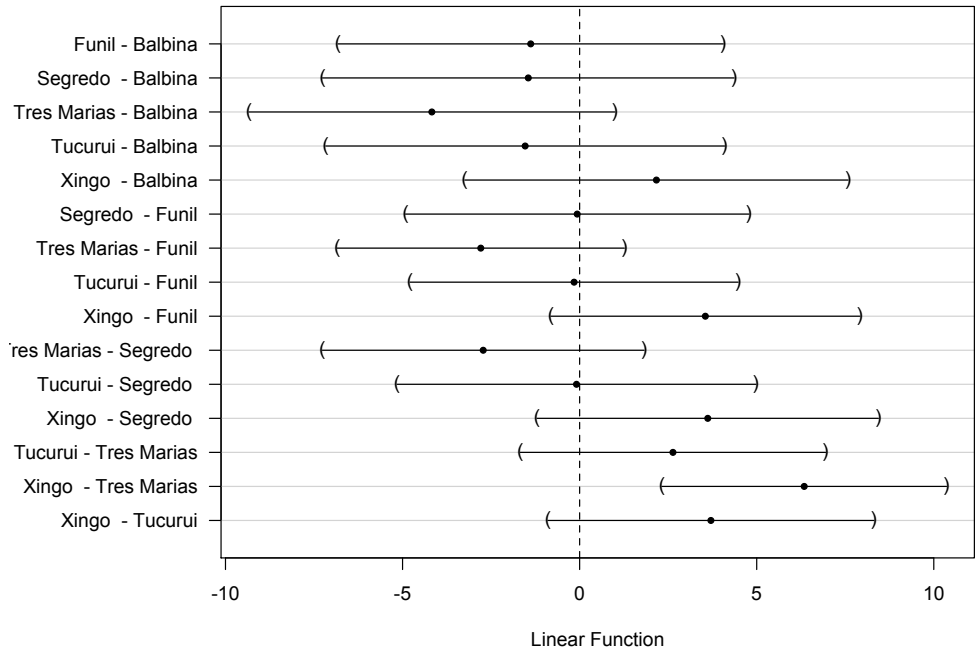

Figure 21 - Tukey multiple comparisons with the six reservoirs studied (Means with 95% Family-wise confidence level.).

3.3.2 Distribution microbial communities and physicochemical variables

We found that relative abundance of methanogens and methanotrophs varied along different zones of the hydroelectric reservoir (riverine, transition and lacustrine zones); also each reservoir show different patterns based in the rainfall index, morphology or dissolved oxygen values (see figure 24). For example results from microbial abundance in Xingó, Segredo and Três Marias suggest seasonality for *pmoA* and *mcrA* copy numbers (Figure 22 and 23). Likewise cluster analysis of genes difference (*mcrA-pmoA*) shows groups in relation to dissolved oxygen. Values quantified in all water and sediment samples ranged between 10^4-10^{10} copies ng-^{1} DNA for *pmoA* and *mcrA*, in the six reservoirs.

Relative abundance of *mcrA* gene revealed that number of methanogenic microorganisms tends to be higher in sediments than in the water column except for some points in Tucuruí and Funil (Figure 14 and 15). Copy number of *mcrA* in the water column ranged between 10^4-10^{10} in the sediment and in some samples there was no *mcrA* detection. For methanotrophic microorganisms, the higher abundance values (Copy number of *pmoA*) were found in in the water column from Funil, Segredo, Tucuruí and Balbina. In Tucuruí, the sediment showed the greatest amounts of *pmoA* copies, but in the other reservoirs abundance was fairly balanced in all layers: sediment bottom water and surface water (figure 22 and 24).

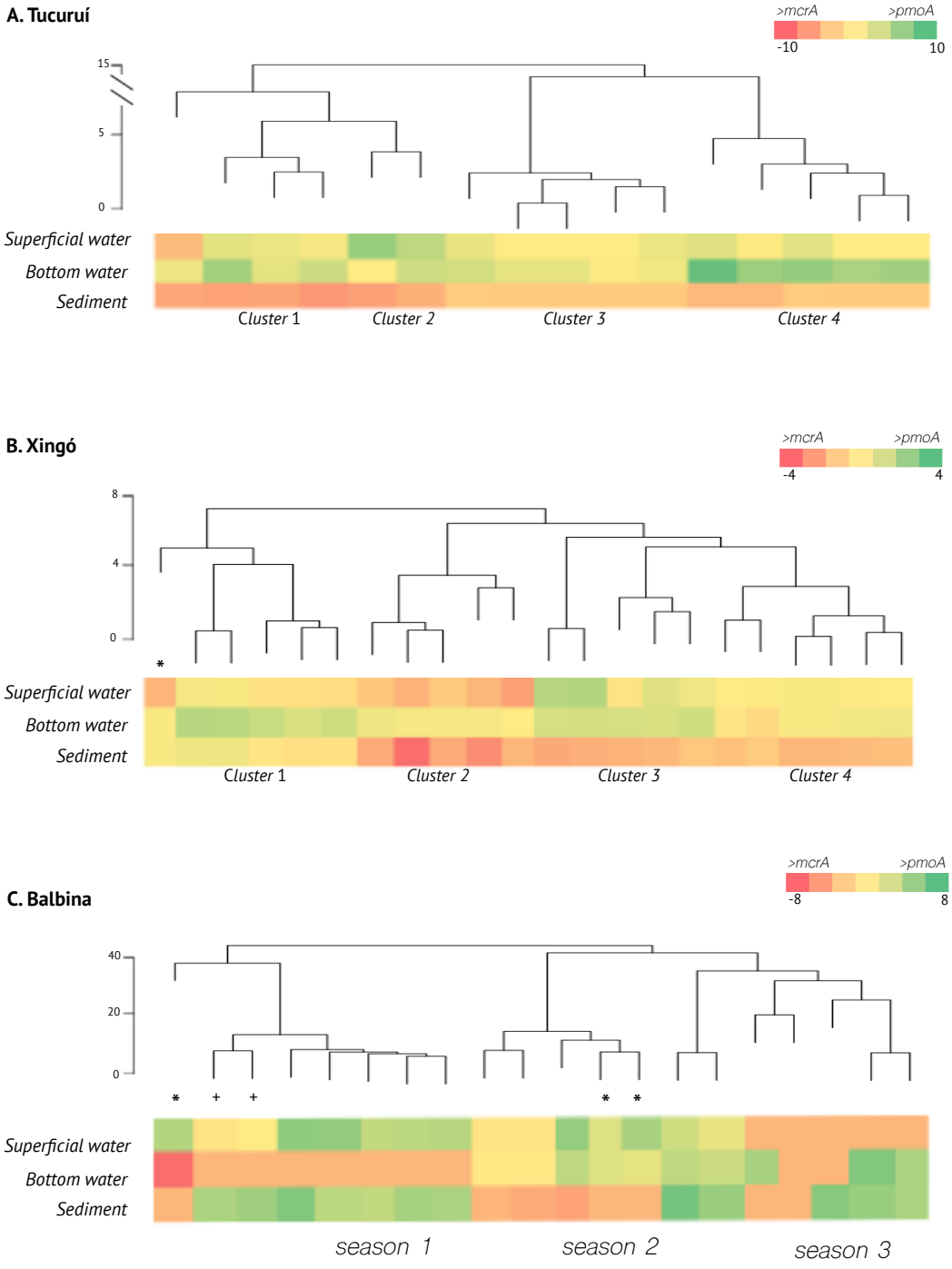

Figure 22 - Cluster analysis of abundance data for genes related to functional processes, *mcrA* and *pmoA*.

Higher abundance of ***mcrA*** is indicated by increased red color intensity. Higher abundance of ***pmoA*** is indicated by increased green color intensity. On the x-axis is the season or cluster number of grouped profiles. On the y-axis, samples were ordered according to layer in the reservoir. The color bar on the right side denotes the scale of difference between the two genes. **A) Tucuruí:** clusters were mainly by different areas in the reservoir**. B) Xingó:** season and reservoir areas outlined clusters; the asterisk indicates a pattern, which does not fall within the group classification defined for this reservoir. **C) Balbina:** most profiles are grouped into clusters relating to three periods of sampling with exception of some collected in the last season (*) or collected in distant places (+).

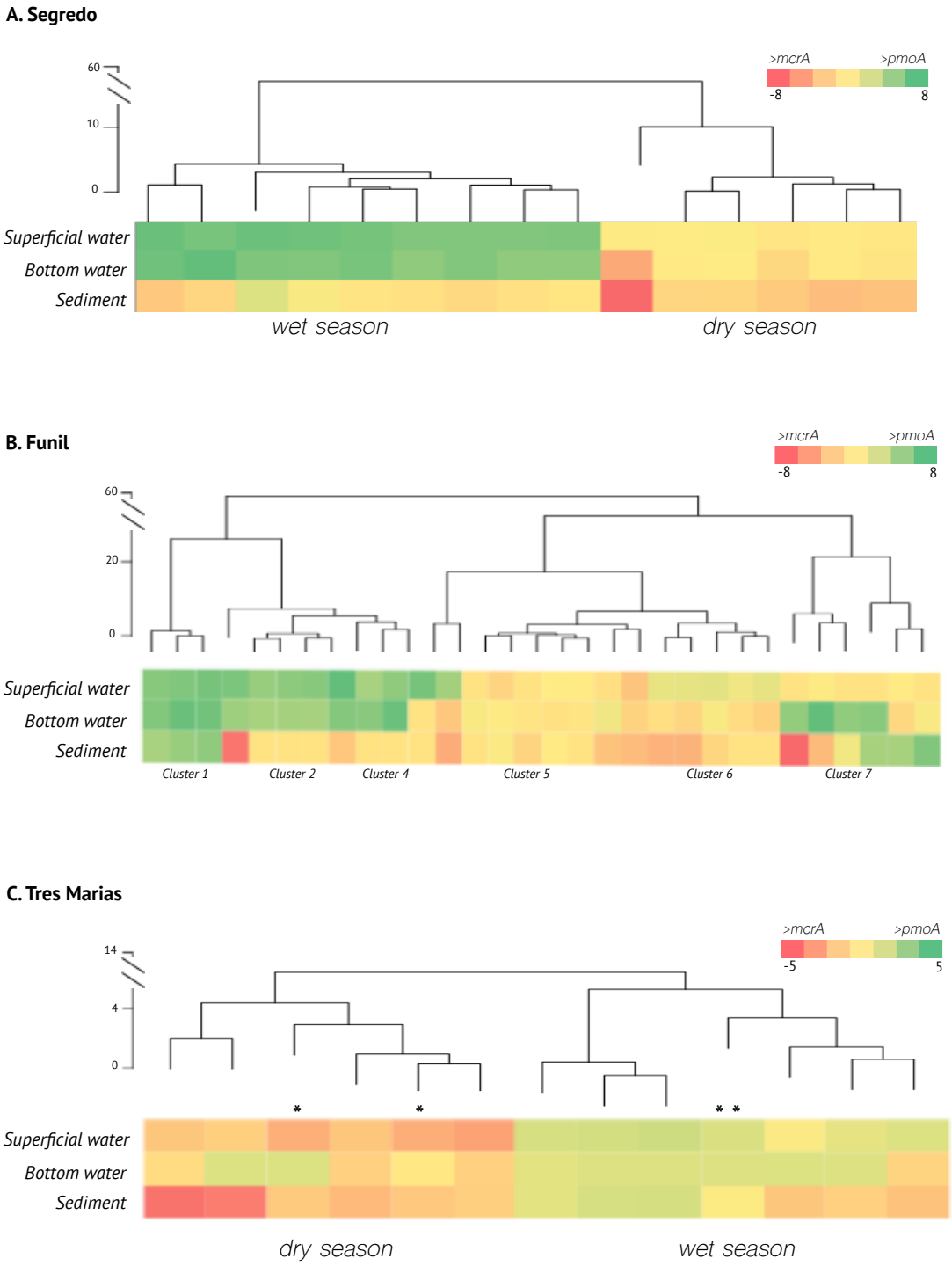

Figure 23 - Cluster analysis of abundance data for genes related to functional processes, *mcrA* and *pmoA*.

Higher abundance of ***mcrA*** is indicated by increased red color intensity. Higher abundance of *pmoA* is indicated by increased green color intensity. On the x-axis is the season or cluster number of grouped profiles. On the y-axis, samples were ordered according to layer in the reservoir. The color bar on the right side denotes the scale of difference between the two genes. **A) Segredo:** clusters were classified by dry or wet season**. B) Funil:** clusters were defined by reservoir area an season **C) Três Marias:** most profiles are grouped into clusters relating to dry or wet season with exception of some samples (*) (**).

PCA indicate that the *pmoA* copy number is positively related to *mcrA*, this confirms the methanotrophs dependence on CH_4 supply as carbon source, in sediment and water samples (see figure 15). For each reservoir we observed some variables (dissolved oxygen, temperature and pH) that are positively related to microbial groups studied. Sample distribution according to the PCA was heterogeneous for Três Marias and Segredo; compared with Funil and Xingó (the reservoirs located in the east) that present a homogeneous pattern. Figure X also shows two groups in samples distributions from Balbina and Tucuruí, as the same of cluster analysis. When data for all reservoirs were analyzed together, the relationships between microbial abundance and dissolved oxygen, temperature were still significant in sediment and column water (See figure 24).

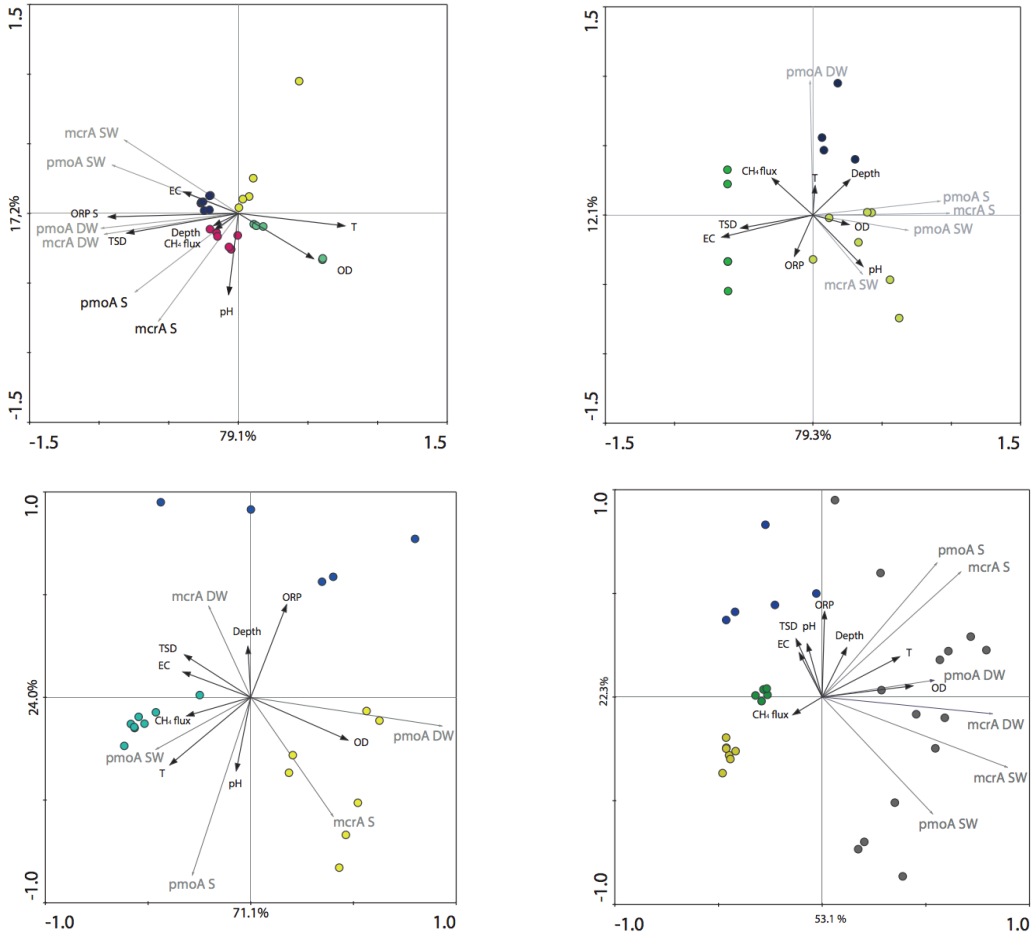

Figure 24 - Principal component analysis (PCA) of Xingó (A), Tucuruí (B), Balbina (C) and Funil (D).

Abundance of *mcrA* and *pmoA* genes in sediment samples (*mcrA S* and *pmoA S*), surface water (SW mcrA and pmoA SW) and bottom water (DW mcrA and pmoA DW). The physical variables on the graph are represented by arrows: Electrical Conductivity (EC), Potential Redox (ORP), Dissolved Oxygen concentration (DO), pH, Temperature (T), Total Dissolved Solids (TDS) CH_4 flow surface water column (CH_4 flux) and depth of the pickup point (depth). Each point represents the number of copies per sample in relation to physical and chemical variables

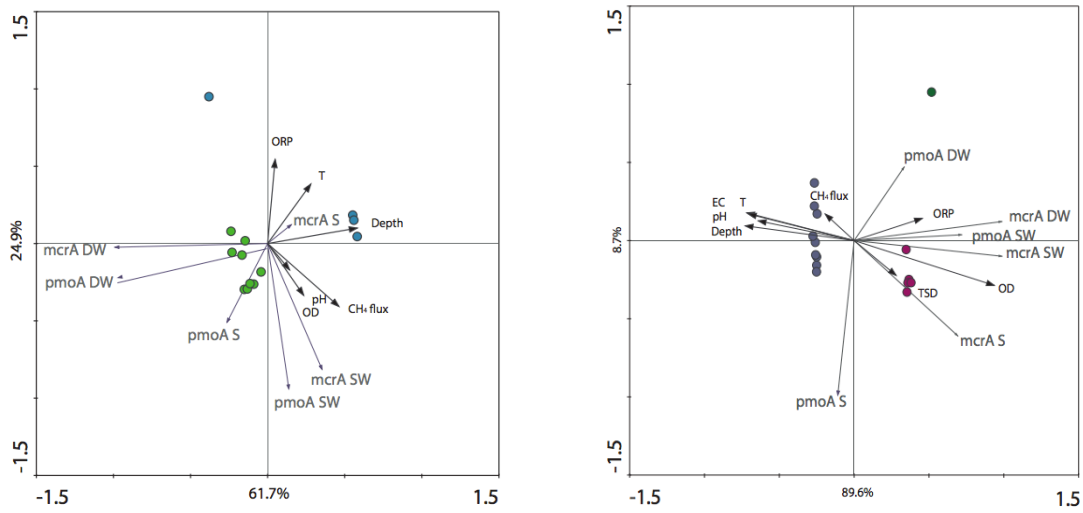

Figure 25 - Principal component analysis (PCA) of Segredo (A), Três Marias(B).

Abundance of *mcrA* and *pmoA* genes in sediment samples (*mcrA S* and *pmoA S*), surface water (SW mcrA and pmoA SW) and bottom water (DW mcrA and pmoA DW). The physical variables on the graph are represented by arrows: Electrical Conductivity (EC), Potential Redox (ORP), Dissolved Oxygen concentration (DO), pH, Temperature (T), Total Dissolved Solids (TDS) CH_4 flow surface water column (CH_4 flux) and depth of the pickup point (depth). Each point represents the number of copies per sample in relation to physical and chemical variables

3.3.4 Correlation

We found significant correlation (coefficients > 0,6) between *mcrA-pmoA* abundances with methane emitted (at water-atmosphere interphase) in the six reservoirs studied (Figure 26, 27 and 28). The correlation was positive for samples from **Funil**, **Xingó**, **Tucuruí,** and **Balbina**; but for **Três Marias** and **Segredo** was observed negative relationship. For cases of positive correlation, graph can be read as when the number of methanogenic organism is greater than methanotrophic; the emission of methane tends to be higher (figures 26 and 27). In the opposite case: when number of methanotrophic exceeds the number of methanogenic organisms, methane emitted into the atmosphere will tend to be lower (figure 28).

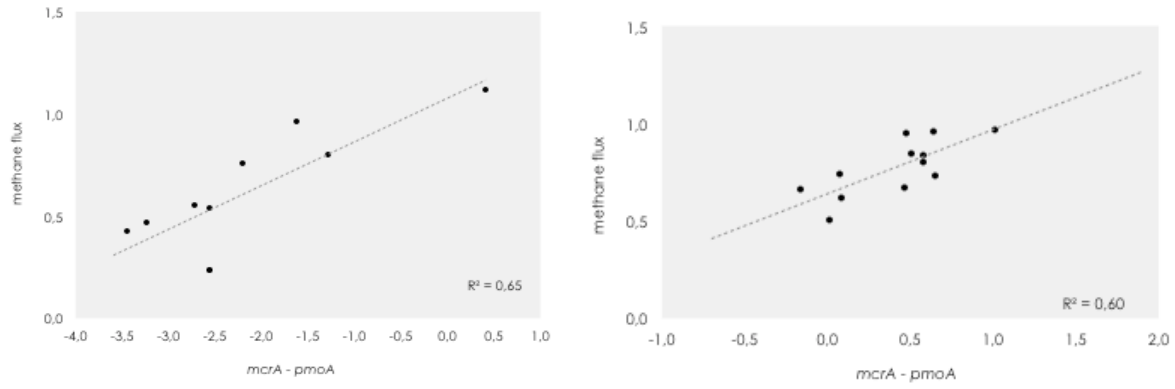

Figure 26 - Simple regression analysis with samples from Xingó (A) and Funil (B).

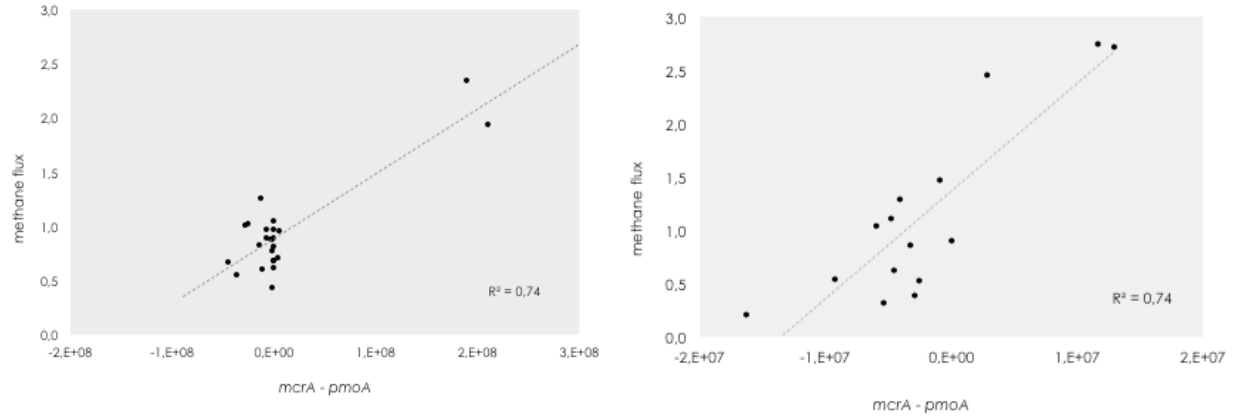

Figure 27 - Simple regression analysis with samples from Balbina (A) and Tucuruí (B).

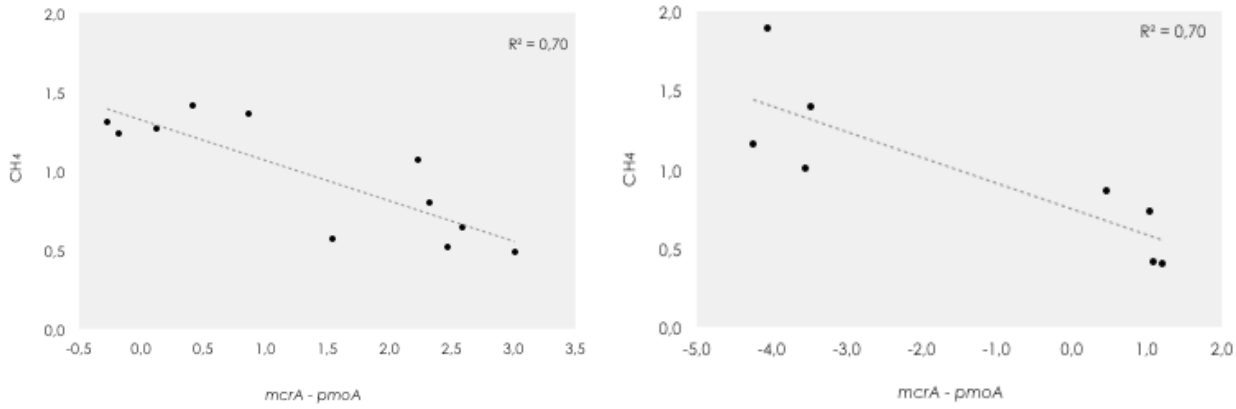

Figure 28 - Simple regression analysis with samples from Três Marias (A) and Segredo (B).

3.4 DISCUSSION

3.4.1 Methane emissions

Data from this study has shown that hydropower reservoirs emit methane (diffusive fluxes) in lower quantities when compared to other works in tropical an equatorial regions (Demarty and Bastien, 2011). Those differences in measurements may be a response to the influence of several factors, including temperature, point depth; wind system, sunlight, physical and chemical water parameters and the operating dam system (Rust, 2015). Methods used by Santos et al., (2008) results in higher fluxes that those measured with floating chambers with laboratory analysis. However this method, used to obtain the data for this work, is more precise and the standards used are closer to the range of actual measurements (Lambert and Fréchette, 2005).

According to MME data (2014), CH_4 fluxes in the sediment-water interface were generally much higher when compared to measurements in the water-air interface, in all reservoirs studied. This is an indication of CH_4 oxidation process in the water column. Only methane diffusive fluxes were analysed in this work, considering that the object of study was the relation of methanogenic and methanotrophic communities with the final methane emission to the atmosphere. Bubbling fluxes (another major pathways of methane emission) were not considered, as a proportion of methane bubbles formed in sediment are dissolved in the water column and the oxidized (Guérin et al., 2006).

3.4.2 hydrological features influencing microbial communities

Ecology in reservoirs is commonly compared to lakes and rivers (Thorton, 1990); nevertheless in rivers microbial community panthers respond more to spatial parameters (Read et al., 2015) and in lakes methanogenesis and methane oxidation depends more on physic-chemical factors that also affect methane transportation thought water column (Borrel et al., 2011). Our data from reservoirs (see figures 23 and 24) shows that dissolved oxygen has a strong correlation with the microbial groups studied (mainly with methanotrophs), in all kind of samples: sediment, surface and bottom water. We also found that the rainfall index can be one of the factors that shape patterns of methanotrophic and methanogenic distribution. These two factors (oxygen and rainfall index) are closely related to hydrological features (Thorton, 1990); and the discussion bellow shows how this can simultaneously influence the ecology of microorganisms associated with methane cycle.

Tucuruí was the biggest reservoir studied, which microorganism's distribution responds to three zones defined by Cluster 1, 2, 4 (Figure 21). The collection points grouped in each cluster can be classified in riverine, transitional and lacustrine areas, which present particular dissolved oxygen values (Cole, Hannan, 1990). Most of sediment samples within lacustrine areas (cluster 1) present more methanogenic microorganism in relation to methanotrophic organism, as occurs in lakes by anoxic conditions (Frenzel et al., 1990). Transition zone (cluster 4) present more methanotrophic communities in bottom water samples, hydrodynamics may allow greater flow of oxygen favouring these microorganisms. Likewise greater retention times than the riverine zone stimulates the consumption of methane produced in sediments, before their diffusion to surface. The *mcrA*: *pmoA* taxa are homogeneous in the riverine area, thanks to the dynamic transport mechanisms as lower retention times of water.

As well as has been reported in Tucuruí by Graças et al., (2011) high abundance of methanogenic archaea was found in Xingó in the water column (Where more oxygen is expected compared to sediment) (Cole, Hannan, 1990)**.** These microorganisms are very sensitive to oxygen however Grossart et al., (2011) and (Bogard et al., 2014) demonstrated the existence of active methanogenesis in the water column of some lakes. Some authors also showed that methanogenic archaea are able to survive in oxic environments such as dry soils (Angel et al., 2012).

In Xingó, distribution of microorganisms from methane cycle appears homogeneous maybe by the operation type of hydroelectric plant (ROR); based on the cluster height scale (max 8) and the *mcrA* - *pmoA* scale (-4 to 4) An interesting pattern is also observed in cluster 2 (and the sample marked with an asterisk): there is a high abundance of methanogenic organisms in samples of surface water, at the level where lower values of these organisms were expected. Most of these collection points have a depth less than 10 m (and are in the riverine area). At this distance between the sediment and surface water layer advection and turbulence are frequent, what might explain the amount of methanogenic organisms found in surface water samples. Two sampling points with the same profile of microbial distributions are located near to the dam, in a period influenced by reservoir outflow operation.

Similar pattern was observed in Balbina, in the cluster named as season 3 (period with more precipitation). Hydrological patterns associated to climatological events as precipitation affect the transport of organic matter to sediments as well as the direct transport of soil and water layers. These transport processes may be the cause of the high methanogenic organism's number found in those samples from surface water.

Samples grouped in season 1 and 2 clusters were from different samplings made in drier periods. Balbina is one of the reservoirs that present lower values of o dissolved oxygen compared with the to other systems, favouring water column the stratification (MME, 2014). For this reason the number of methanogenic organisms, that grow best under anoxic conditions, were more abundant in this reservoir.

The effect of seasonality is clearer for Três Marias: in samples of dry period there is more abundance of methanotrophic groups in all compartments, sediment and water. The opposite occurs for the rainy season where the number of methanogenic organisms is higher. In lakes it has also observed the same seasonality effect. Methane formation changes with the raise of water level that covers a proportion of the previously exposed littoral areas with vegetation (during the dry season) (Yang et al., 2014). This water input carries nutrients (organic carbon, P and N) that will promote the growth of methanogenic organisms, after degradation of organic mater into simpler molecules by other organism.

Cluster analysis for the Segredo reservoir also shows two principals groups, which correspond broadly to hot or cold periods. Climatological events seem to be a driver for abundance and distribution of methanogens and methanotrophs in some Brazilian reservoirs. However for this system, differences between collection periods are based on temperature and not the precipitation patterns (which in this region is constant throughout the year). In the hottest periods there are more methanotrophic organisms compared to the number of methanogenic microorganisms. Methanotrophs are limited by their main substrates methane and oxygen and potentially even by space (Ho et al., 2011). If the carbon cycle is more productive at high temperatures (Davidson, Janssens, 2006), there will be more available substrate (CH_4) to be oxidized resulting in increased number of methanotrophic organism.

For reservoirs as Funil, with a particular morphology (see figure 14), is difficult establish a causal relationship or define the main factor among many that shape microbial ecology: hydrologic regime, temperature, wind, depth, oxygen concentration, dam closing time, and carbon input (Rogério et al., 2013). Funil also present great influence of human activities such as agriculture and livestock in points near the lake (MME, 2014). Morphology and allochthonous inputs influence the distribution of oxygen in the reservoirs (Cole and Hannan, 1990), and some clusters are grouped according to the difference of this variable. When there is more oxygen in the water column, there is a balance (one to one) between copy number *mcrA* and *pmoA* (see clusters in figure 22).

3.4. Latitude and distribution of methanogenic and methanotrophs communities

Results suggest that the distribution of microorganisms could be related to the latitude of the reservoir. Just as other authors have reported the influence of latitude in the amount of Emissions of greenhouse gases and food webs in reservoirs (Barros et al., 2011; Maeck et al., 2013; Roland et al., 2010). In this study, each reservoir revealed many particularities, but distant reservoirs from equatorial region respond more to a seasonal distribution associated with methane cycle. In addition to the climatological differences in these latitudes, geological patterns influence reservoir water quality. For example, the trend of values of total solids dissolved is greater the reservoirs located in northern Brazil (Xingó, Balbina, and Tucuruí)

3.4.4 Microbial communities regulate methane fluxes

These results support the postulate that CH_4 flux rates are mainly controlled by methanotrophs abundance; following in lowest emissions of greenhouse gases than expected on tropical hydropower dams. In peat bog samples, was found a similar outline, but were quantified microbial activity (and not only gene abundance) using transcripts of functional genes (Freitag, Prosser, 2010). Sharma et al. Correlation analysis revealed that both were significantly correlated positively with methane production potentials. Obtained similar results when studying composting. They observed that net ratio of *mcrA: pmoA* copy numbers is Higher when surface CH_4 flux was high.

This relationship suggests that the abundance of methanotrophs cover the amount of methane produced by the methanogenic organism in these samples. These two reservoirs present dissimilarities in water temperature one of the controlling parameters in the methane diffusion and methanotrophic activity (Gudasz et al., 2010). This would explain the singular pattern in relation to other systems. The three cases of correlation reveal that just a fraction of the amount of CH_4 produced is emitted to the atmosphere. Methanotrophs occurrence in sediments and water column justify why methane emissions measured in those tropical dams are lower than the potential for emission predicted (Yvon-Durocher et al., 2014). An environment is a CH_4 source when CH_4 production by methanogenic archaea is greater than its consumption by methanotrophic bacteria. On the contrary, if methane oxidation is greater than its production, the environment is a sink for methane. A change in the equilibrium between sources and sinks can lead to increased atmospheric emission. Another important observation is the negative intensity values found, showing that the dams can be GHG gases sinks to Instead emitters.

3.4.5 Hydropower operational planning for climate change

Most greenhouse gas assessments ignore that hydroelectric reservoirs can sequester organic carbon in sediments (Stallard, 1998). CH_4 derived carbon could be transferred in substantial amounts to higher trophic levels. Sector considering the construction of hydroelectric power plants in the Amazon region, energy security requirements, projected economic growth and climate change feedbacks (Alameda Prado et al., 2016).

Global climate change might have a larger effect in terms of precipitation patterns than in terms of temperature. Many paleoclimate proxies show large millennial changes in Amazonian precipitation and Amazon River outflow during the Late Pleistocene and Holocene (Govin et al., 2014, Manabe et al., 2004, Maslin et al., 2000), which likely plays a large role in regulating microbial activity in methane cycle.

In these reservoirs this variable may have an influence abundance methanogenic communities as well has been found in other systems like anaerobes reactors and lakes (Liu, Whitman, 2008). Most methanogens grow optimally under neutral to slightly alkaline conditions (pH 6.8– 8.5). In lakes, low pH provides a selective advantage for other organisms that reduces CO_2 and produces acetate. This phenomenon limited growth of methanogenic that use H_2/CO_2 as substrate. Similar to temperature this parameter is related to the availability of substrates for metabolic processes, Which Affect the relative abundance of these organisms. Our results show that pH does not Influence in the same way methanotrophic communities. Different works report that methanotrophs are well adapted to pH conditions of their environment. This leads to inaccurate characterization of the effect river damming on the carbon cycle (Mendonça et al., 2012). Dark carbon fixation amounted up to 80% of the total heterotrophic bacterial production (Santoro et al., 2013).

For example, the potential for water column CH_4 oxidation is greatly limited when river water is pumped through hydropower turbines, which expedites the release of CH_4 to the atmosphere and reduces its residence time in the river. Most of these hydropower plants are planned to operate as run-of-the-river, which in contrast to traditional reservoirs are characterized by the short residence time of the water and a small increase in the water level. At present there is limited knowledge about CH_4 cycling in such reservoirs.

4 CONCLUSIONS AND FINAL REMARKS

Differences in carbon entry into new reservoirs modify the microbial community structure resulting in potential emissions of methane. A methane increase was observed after incubating soil samples (collected in areas intended to reservoirs) with different methanogenic carbon sources. However, the amounts of methane were not significant when compared between the carbon sources.

Metabolic pathways associated to acetate seem more productive, since the methane produced was the highest in microcosm with this carbon source. Bacteria and Archaea diversity changes in response to organic matter differences in the flooded samples.

Assessment of stress (anoxic conditions and carbon source changes) changes in identity of the dominant species and the community structure after flooding soil simulations. As expected shifts in microbial community composition following the exposure to a novel environmental regime lead to increased productivity (methane production, in the case of methanogenic archaea) and ecosystem performance.

The strong correlation between relative abundance of microbial groups and methane fluxes in the air–water interface, demonstrated that relations in gene abundances correspond with methane cycle in the six ecosystems studied. Geographical location influences methane emissions and microbiology related to carbon fixation in aquatic environments. According to multivariate analysis, oxygen, geophysical and climatological parameters shape the balance of microbial communities related with methane cycle. Methanotrophic organisms consume a proportion of methane product of organic matter decomposition resulting les CH_4 emitted into the atmosphere. With hydropower operational planning (based in microbial ecology information) will be possible manage quantity of methane emissions.

The balance between methanogenesis and methane oxidation, quantified by *mcrA* and *pmoA* abundances, will result in different values for methane fluxes at the air-water interphase. Also if methanotrophic organisms consume a proportion of methane product of organic matter decomposition; the amount of CH_4 emitted into the atmosphere could be managed with hydropower operational planning (based in microbial ecology information).

Efforts have been invested in order to identify potential mitigation strategies for the CH_4 emission in hydropower reservoirs and prevent higher consequences for the climate change scenario. The challenges for future hydropower projects are settles the reduction of emissions greenhouse gases and guarantee the supply of energy demands, sustainably.

The Energy Research Company estimates that Brazil needs 6.350 megawatts of new electricity generation per year between now and 2022. Today the country obtains 70% of their energy from hydroelectric power plants. Hydropower is an attractive energy option for many reasons. It is cheaper than thermoelectric power and most other renewable forms of electricity, can provide energy at scale more easily and with fewer disruptions than wind or solar, and can potentially provide electrical energy with lower levels of greenhouse gas emissions than thermoelectric energy, although its effect on methane production could counteract this benefit.

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ANNEXES

A

LIST OF WORKS PRESENTED IN SCIENTIFIC EVENTS AND PUBLICATIONS IN COLLABORATION

Castro WD, **Torres-Ballesteros AM**, Nakayama CR, Melo IS, Pellizari VH, Silva A, Ramos RTJ. 2014. Draft genome sequence of Haloferax sp. strain ATB1, isolated from a semi-arid region in the Brazilian Caatinga. Genome Announc. 2(4):e00812-14.doi:10.1128/genomeA.00812-14.

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B

PROJECT FOR THE IPCC AWARD- SCHOLARSHIP APPLICATION

ipcc
INTERGOVERNMENTAL PANEL ON climate change

Our ref.: 5287-15/IPCC/SCO

Ms Adriana Maria Torres-Ballesteros
Oscar Freire Street, 1606. apt 112B
Brazil

GENEVA, 14 July 2015

Dear Ms Torres-Ballesteros,

I refer to your application to the 3rd round of the IPCC Scholarship Programme. I am pleased to inform you that your application has been successful.

A formal agreement document with the details of your scholarship award and the conditions to be fulfilled by all the parties involved in your scholarship will be sent for your signature in due course.

You will also receive an invitation letter from the Prince Albert II Foundation of Monaco inviting you to attend the official award ceremony to be held in Monaco on **2 October 2015.**

Please contact Mr. Mxolisi Shongwe (email: ipcc-sp@wmo.int), the desk officer responsible for the Scholarship Programme at the IPCC Secretariat, in case you need more information.

I would like to express my heartfelt congratulations to you for this award and looking forward to contributing to building up your career.

Yours sincerely,

(Carlos Martin-Novella)
Deputy Secretary of the IPCC
Officer-in-Charge

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WMO UNEP

Hydropower and greenhouse gases emission: methane formation in tropical reservoirs

Metabolic fluxes in sediments with different carbon input.

Adriana Torres-Ballesteros

ABSTRACT

Hydropower has the potential to mitigate climate change, although carbon footprint of this renewal energy source is still questioned. Methane emissions seem to be higher in tropical areas and regulation of carbon input in reservoirs is proposed to be a strategy to reduce green house emissions. Carbon input in reservoirs could influence methane production by microorganism, and consequent emission to the atmosphere. Here, we propose to evaluate changes in methanogenic microbial communities in different levels of carbon input (low and high). In order to measure the effect of rising organic matter in flooded area, two different sediments from Brazilian reservoirs will be analyzed using stable isotope probing (SIP) and next generation sequencing. The study will elucidate the importance of carbon input level in this system, which is key information to develop mitigation strategies for methane emission, while stimulating hydroelectric development.

1. Introduction

Research and development in energy efficiency could help in adaptation and mitigation strategies of climate change while enhancing the prospect of achieving sustainable growth. Climate change affects the function and operation energy infrastructure (as hydroelectricity) due to increasing of water demand in the coming decades, primarily because of population growth (Bates *et al*., 2008).

Hydropower is one of the main energy sources in tropical areas. In Brazil, 67% of the power demand is supplied by hydroelectric plants (ANEEL, 2015). This renewal energy has the potential to mitigate climate change, but carbon footprint of hydropower reservoirs has been questioned in the last years (Hu & Cheng, 2013; Fearnside & Pueyo, 2012). Although methane is known as one of main greenhouse gases, the emission of this gas is still not included in assessments of environmental impact of hydroelectricity (Li & Lu, 2012). In this regard, recent works have tried to define and quantify methane emission factors in hydropower reservoirs (Roland *et al*., 2010; Barros *et al*., 2011; Maeck et al., 2013).

Biological processes related with methane formation remains unexplored in reservoirs sediments. In tropical areas, where carbon cycle is more productive and methane emissions seem to be higher than temperate areas (Yang *et al*., 2014). Efforts have been invested in order to identify potential mitigation strategies for the CH_4 emission in these systems and prevent higher consequences for the climate change scenario.

Methane fluxes result from the balance between two microbial process: CH_4 production (methanogenesis) and CH_4 consumption (methane oxidation). Organic carbon sources (carbohydrates, long-chain fatty acids and alcohols) are mineralized and a proportion of substrates, obtained are channeled in to CH_4 production (Lue & Withman, 2008) by anaerobic microorganisms from Archaea domain called methanogens (Conrad, 1999). The methanogenesis is very well described in freshwater sediments (Borrel *et al*., 2011), and recently it has also been detected in the water column (Bogard *et al*., 2014). The amount of gases that is transfer to atmosphere also depends on a variety of physical factors (as geometry and hydrodynamic of reservoirs) (Bambace *et al*., 2007; Ometto *et al*., 2013) (see figure 1).

Methanogens diversity and metabolic capabilities are usually associated with substrate chemistry (Conrad, 2009). Similarly, other microbial communities are related to composition, quantity, and variety of carbon molecules in the environment. (Wang *et al*., 2013; Hernandez & Hobbie, 2010). Therefore, decreasing organic carbon input could be a key element in reducing methane emissions from reservoirs (Bergier, Ramos & Bambace, 2014). However, to date no study has been performed to verify the effect of carbon concentration on methanogen communities and CH_4 fluxes in reservoirs.

Among the innumerous methods to study environmental microbial communities the Stable Isotope Probing (SIP) has the great advantage to target the members actively involved in the metabolism of a specific substrate. A substrate highly enriched with a stable isotope (e.g.,^{13}C) is incubated with an environmental sample to detect metabolically active organisms inside complex microbial consortium (Offre, Spang & Schleper, 2013). From genomes sequences and biochemical data it is possible to create network models to elucidate metabolic capabilities. A metabolic network links molecular mechanisms (reactions) to associated molecular components (enzymes, substrates, and products) in defined system conditions (Durot, Bourguignon & Schachter, 2009).

Carbon input differences in reservoirs can modify methane formation at community and molecular level (resulting in changes of methane emission to the atmosphere). Metabolic capabilities of organisms associated with formation of these gases could be different in response to organic matter changes in the flooded area. The aims of this work are (i) to measure potential methane formation in Brazilian reservoir and (ii) to evaluate changes in methanogenic Archaea metabolism at different carbon source conditions. Two different sediments from Brazilian reservoirs will be incubated with ^{13}C-labeled substrate, analyzed using stable isotope probing (SIP) and next generation sequencing.

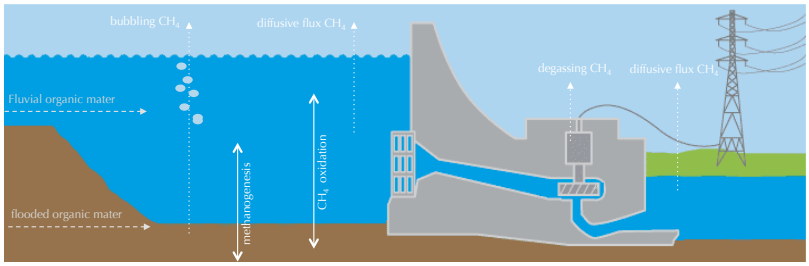


Figure 1. Methane cycle in hydropower reservoirs. Fluvial and flooded organic mater (soil, plant material, wood) is degraded to simplest compounds that are substrates for methanogenesis. Methane formation occurs mainly in sediments. Bacteria in the superficial sediment and column water oxidize a proportion of methane. The other part of methane is emitted to the atmosphere by bubbling, diffusive flux and in a degassing process at the dam. (Adapted from concepts in Goldemfum, 2010; Borrel *et al.*, 2011 and Bogart *et al.*, 2014).

How is my research beneficial and to whom?

A proportion of research related to climate change is driven by the need to mitigate effects of rising global temperatures. Existing information of green house emissions from hydropower is not enough and there are difficulties in making decisions in energy infrastructure investment. The information generated from this project would provide scientific evidence to take actions to mitigate methane emission and stimulate hydroelectric development in tropical regions (one of the main sources of energy).

This research also will bring benefits to the scientific community interested in energy production and climate change. The results may provide some keys to design strategies in which renewable resources are used and emissions of greenhouse gases are minimized. Microbial ecology techniques will be used to test hypothesis; studying microorganisms in these environments will be an innovative contribution in this field.

The knowledge gained in this project can be transmitted to the research institute where currently I'm doing my PhD (in **Brazil**). Also I could lead seminars in **Colombia** through agreements with entities such as UNIAGRARIA (Agronomic University of Colombia) and CORPODIB (Corporation for Industrial Development of Biotechnology and Clean Production). Scientific directors of this institutions have expressed their interest in develop this kind of collaboration (see appendix 1).

2. Hypothesis and objectives

Carbon input differences in reservoirs can influence methane formation at community and molecular level. Metabolic capabilities of organisms associated to methanogenesis could be different in response to rising organic matter in the flooded area. The objectives of this work are (i) measure potential methane formation in Brazilian reservoirs using microcosms and (ii) evaluate changes in active methanogenic metabolism at different carbon source conditions, (iii) designing metabolic fluxes from functional gene sequences.

3. Methodology

Stable isotope probing is a method used for labeling uncultivated microorganisms in environmental samples; microorganisms that consumed substrate with ^{13}C become enriched with the isotope. Labeled biomarkers, such as DNA can be analyzed by molecular (by next generation sequencing) and analytical (by metabolic fluxes) techniques to identify and characterize organisms that incorporated the substrate (Neufel *et al.*, 2007). This project is divided into different phases: Incubation, separation, sequencing and metabolic analysis.

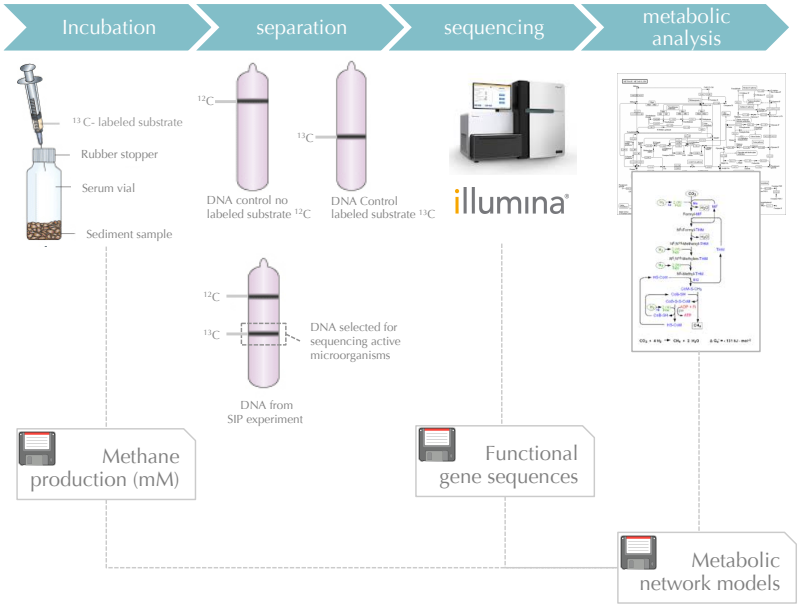


Figure 2. SIP experiment. Incubation: sample is transferred to a microcosm with a target compound labeled with an isotope (^{13}C); metabolically active microorganisms incorporate the ^{13}C. Separation: total DNA is extracted from samples and with ultracentrifugation the fractions are separated and collected separately. Sequencing: With material obtained after precipitation, with DNA contained in the fractions of interest it is possible to analyze the genes-related to metabolic functions and diversity. Metabolic analysis: (Modified from Dumont & Murrell, 2005).

3.1 Samples

We collect sediment samples in Itaipú and Três Marias reservoirs (see figure 3) with a dredge van Veen (AFK 3841). For each sampling point, methane flux measurements were performed ($mgCH_4/m^2/day$) using Marani & Álvala (2007) method. 20 grams of sediment (equivalent to 2 g dry weight) were weighed into incubation vessels (150-ml glass bottles Schott), fluxed with N_2 and stored for futures experiments. Sediments were stored at 4°C and pre-incubations were carried out to identify sediments with highest microbial activities, based on methane production. During processing and preparation of microcosms, samples were always maintained under anoxic conditions at room temperature.

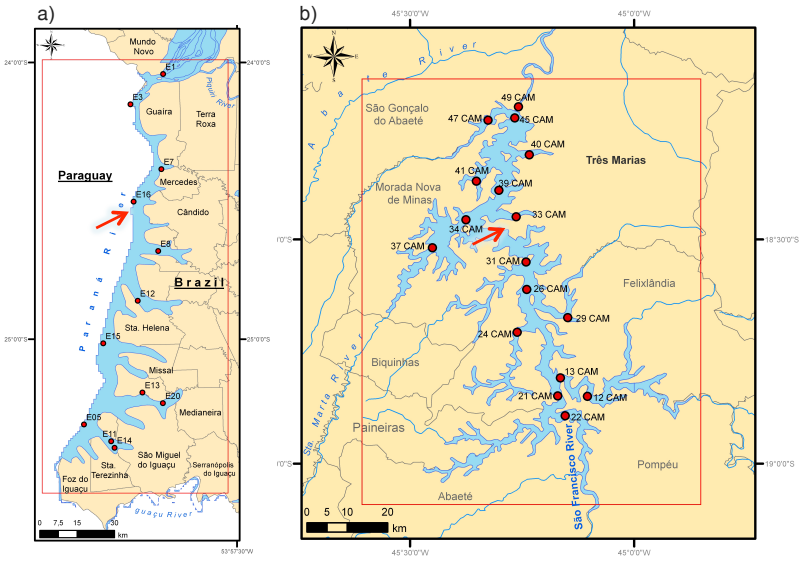


Figure 3. Geographic localization of reservoirs. a) Itaipú reservoir: sediment was selected from E16 point (24°30'9.7"S 54°19'52.0"W), where the highest values of methanogenic activity were detected. b) Tres Marias reservoir: sediment was selected from Cam 33 point (18°33'1,5"S 45°14'28,5"W), where the highest values of methane activity were detected.

3.2 Incubation

Stored sediment will be distributed in 15 ml aliquots in 70ml vessels sealed with butyl rubber stoppers. Either fully ^{13}C-labeled or unlabeled (for control experiment) carbon source is added to the vessels, using N_2-purged gastight syringes. Carbon source concentrations in experiments (10 mM acetate; 10 mM methanol) should be higher than natural conditions to prevent substrate availability limitations in SIP experiment.

During incubation period, aliquots are taken for methane measurements in a gas chromatograph with flame ionization detection. Methane concentrations are determined from a calibration curve, prepared with 99.5% methane. The values are expressed in m moles of CH_4 (Nakayama *et al*., 2011). After time incubation will be done DNA extraction from samples using extraction kit: DNA Power Soil (MO BIO Laboratories, Solana Beach, USA).

3.3 Separation (**ultracentrifugation and fractioning**)

^{13}C-labeled DNA from metabolically active community will be separated by ultracentrifugation using a gradient of cesium trifluoroacetate. Total DNA will be transferred to 6-ml polyallomer UltraCrimp tubes, sealed and spun in a rotor using an ultracentrifuge at 130 000 g, 20 °C for 65 h, and gradients fractionated after ultracentrifugation (Lueders *et al*., 2004).

Syringe pumps are ideal to fractionation because they provide smooth delivery of displacement liquid, which minimizes disruption of the gradient (obtained after centrifugation). Fractions are collected from the ultracentrifuge tube drop wise (Neufeld *et al*, 2007) (see figure 4: fractionation scheme). DNA is precipitated from fractions with isopropanol and eluted in Milli-Q water. Then DNA concentration in each fraction is measured using PicoGreen kit (Invitrogen) and density quantified with a refractometer (Lu *et al*., 2005).

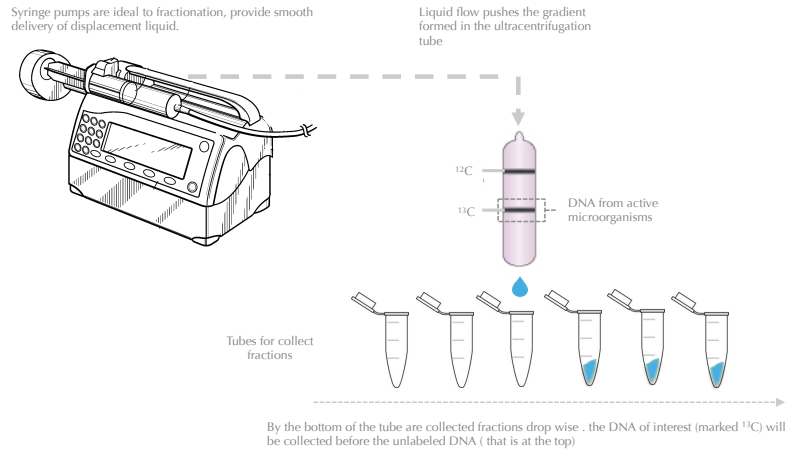


Figure 4.Fractionation.

3.4 Illumina® sequencing

Shotgun metagenomics is a method that enables to evaluate genes in all organisms present in a sample; also it provides a means to study uncultivable microorganisms that are difficult to analyze. From this data it is possible infer metabolic potential and biogeochemical cycles associated with microbial community.

In this project, Hiseq 2500 platform (Illumina) will be used for sequencing by outsourcing services in LaCTAD laboratory (High Performance Technologies Central Laboratory in Life Sciences), located at Campinas- Brazil. The sequencing will be held in a pair-end system 2x100pb in one lane with approximately 30 million reads per sample.

For the analyses we will be use MG-RAST, a public source that was specifically developed to handle metagenome data. The user interface provides a means to alter some of the parameters used to compute the functional, metabolic, and phylogenetic reconstruction (this allows more accurately information for different kind of sample). The pipeline produces automated functional assignments of sequences in the metagenome by comparing both protein and nucleotide databases (Meyer *et al*., 2008).

3.5 Metabolic analysis

Metabolic reconstruction is a process where genes, proteins, reactions and metabolites that participate in the metabolic activity of a biological system are identified, categorized and interconnected to form a network (Raman & Chandra, 2009). Among Archaea, methanogens are an attractive model for this analyses because of their utilization of low carbon substrates, metabolic diversity and the availability of detailed information on their metabolism (Feist *et al*., 2006)

From sequences of microorganisms related to methane production it is possible to construct a model of the most probable pathways in methanogenesis. One way to reconstruct metabolic networks from this kind of data is calculating elementary modes in biochemical reactions. It allows enumerating systematically all independent minimal pathways through a network that are stoichiometrically and thermodynamically feasible. For this analysis we will use Metatool, one of the first programs dedicated to this purpose (Von Kamp & Schuster, 2006).

4. Expected results

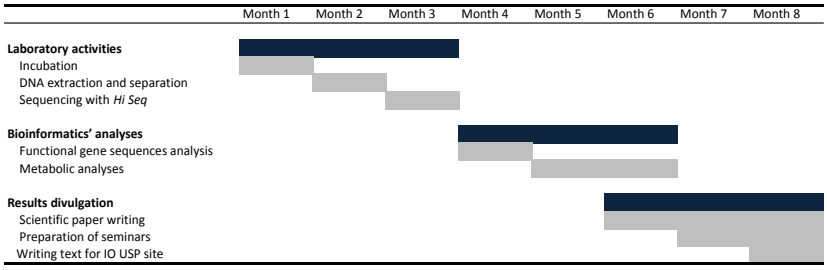
The project results will be submitted to scientific journals in the area of microbial ecology. Additionally, we will publish on the website of the Oceanographic Institute of USP texts explaining to the academic and nonacademic community the importance of this research, The main results expected from this study are:

- Potential methane production in sediment samples with different carbon input.
- Structure, abundance, and metabolic characterization of methanogenic organisms from sediment associated to different carbon input.
- Most probable metabolic fluxes in each carbon input condition in the sediment tested.

5. Ethical considerations

I'm participant of Balcar study (Carbon Balance in Reservoirs), made by the ELECTROBRAS group and ten Brazilian research institutions, including Sao Paulo University (USP) (Gandra, 2014) and Federal University of Pará (UFPA). Professor **Artur da Costa da Silva** from UFPA leads the microbiology group from the study. As a student member of the Balcar, I am allowed to use samples collected in previous years for my PhD thesis and I'm able to access other datasets, as methane emissions in the reservoirs to discuss results that will be obtained in this project.

6. Chronogram



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