Using Extracellular Enzyme Activity as a Pollutant Indicator: a Field Study in Chinchiná River, Caldas – Colombia

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Abstract- **The present study investigated the existence of a relationship between Extracellular Enzyme Activity (EEA) of glucosidase and alkaline phosphatase and pollution by carbon and phosphorous at five stations on the Chinchiná River in the water main and the biofilm (epilithon/episammon) during three seasons (rainy, dry and transition). Specific substrates were used as sources: 4-Nitrophenyl-β-D-Glucopyranose as a carbon source and 4-Nitrophenylphosphate as a phosphorus source. The product of catalysis (4-nitrophenol) was detected spectrophotometrically at an emission of 405nm. The ratio of EEA to temperature and pH was also determined. All sampling stations displayed EEA; however, reported results were higher for phosphatase, specifically in the biofilm, in all seasons; this indicates that certain associated microorganisms in this matrix can act as a multi-enzyme system which allows for easy disposal of substrate and the presence of catalysis. A relationship could not be established to describe EEA in dissolved organic carbon (DOC), because EEA was not detected in water samples from all stations during the three sampling seasons (E1, E2 and E4), because the bioavailability of nutrients attributed to the discharge of domestic wastewater from the municipalities of Manizales and Villamaría. Additionally, the complexity of the links between the monosaccharides which comprise polymers affects the degradation rate of the material, since the enzymes produced by microorganisms in the water prefer to hydrolyze specific regions (regiospecific) of the molecule. Enzyme activity can be affected by the structure of the polysaccharide being degraded. Therefore, some trends, such as those which occur at lower DOC concentrations, exhibit greater EEA. During the dry season, a correlation was found between phosphatase and glucosidase EEA in samples of water related to the concentration of orthophosphates and filtered COD, respectively. This indicates that higher concentrations of orthophosphates result in higher EEA of the phosphatase, and that higher concentrations of CODs result in higher EEA of the glucosidase.**

Keywords- Alkaline Phosphatase; Extracellular Enzyme Activity; Epilithon; Glucosidase; Nitrophenol; Chinchiná River

I. INTRODUCTION

Decomposition is a very complex process which manifests at the community level, and involves multiple agencies operating on different spatial and temporal scales. Primary production is a process which ultimately manifests itself at the level of each autotrophic organism individually, and involves a much smaller fraction of biodiversity. Alternatively, it is extremely complex to analyze microbial fractions, which develop most decomposition processes, and researchers have only very recently begun to develop techniques to study the functioning of these communities, such as extracellular enzyme activity [1].

Most organic matter (OM) consists of either Organic Particulate Matter (OPM) or Dissolved Organic Matter (DOM). Organic matter is relatively recalcitrant (ROM) and of a size that cannot be absorbed by osmotrophic organisms [2]. Except for phagotrophic organisms which directly ingest OM [3], other organisms decompose OM slowly and require its degradation or depolymerization in order to absorb it. This synthesis of molecules can be conducted by photolytic reactions, which are of great importance [4], or by microbial extracellular hydrolytic enzymes with oxidative characteristics that degrade these substances on the outside of the cell [5]. These enzymes are unique to microorganisms, including bacteria, fungi, phytoplankton and protozoa, which comprise a key group in the use of organic matter, which is then transferred to higher trophic levels. Enzymatic action is a critical and fundamental step in the processing of detritus [6]. Enzymatic activity also provides an overall estimate of nutrient requirements by the microbial community as a whole, and therefore the processing of nutrients in the aquatic system.

The OPM and DOM are dominated by high molecular weight compounds [7]. To allow transport across the outer membrane, complex substrates must first be hydrolyzed into smaller molecules [8-9]. This process is performed by extracellular enzymes which enable heterotrophic bacteria to obtain suitable substrates for the incorporation of a diverse set of compounds [10]. Catalysis by extracellular enzymes is the limiting step in nutrient cycle; thus, the extracellular degradation of complex molecules into easily monomers [11-12], affects the remineralization process, as do factors which affect the activity of interrupt production of availability of the nutrients [10].

β-glucosidase (EC 3.2.1.21) is widely distributed in aquatic environments and is primarily associated with heterotrophic bacteria. It has been used as a model according to which the ectoenzyme bacterial degradation of natural polymeric compounds such as carbohydrates and proteins may be studied in the aquatic environment [13]. β-glucosidase is produced by heterotrophic

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bacteria in water and sediment in both freshwater and marine environments. This enzyme exhibits substrate specificity for the hydrolysis of glycosidic bonds of the β type disaccharide of glucose, celuhexose and carboxymethylcellulose [14].

Alkaline phosphatase (EC 3.1.3.1) is a widely studied enzyme found in aquatic environments. Its activity may be observed in bacterioplankton, phytoplankton and zooplankton [15]. The acquisition of phosphorus, particularly in areas in which phosphorus is the limiting factor, is dependent on availability of the enzyme which hydrolyzes dissolved organics. Phosphatases are a phosphohydrolases group which is more intensely involved in the release of phosphate into the aquatic environment [16]. Generally, the phosphatase family contains a variety of enzymes which catalyze the hydrolysis of esters and anhydrides of phosphoric acid [17]. These enzymes are characterized by different constant saturation temperatures and optimum pH levels [18]. Alkaline phosphatase (APA) includes a group of isoenzymes which react optimally in the range of pH 7.6 to 9.6 [16]. The APA catalyzes the hydrolysis of a variety of phosphate esters, including esters of primary and secondary alcohols, cyclic alcohols, phenols and amines, which release inorganic phosphate [10].

The activity of many extracellular hydrolytic enzymes can be accurately measured by fluorogenic models which are available for a group of natural compounds [19]. These model substrates consist of 4-methylumbelliferone (MUF) or 4-nitrophenol, or of molecules bound to other compounds (e.g., glucose, phosphate, amino acids), which are hydrolyzed by the enzyme in a similar manner to polymeric substances. The MUF or 4-nitrophenol released by the hydrolysis is fluorescent to an extent which may be determined fluorometrically, and which indicates the activity level of extracellular enzyme in a sample.

Hence, our research purpose is to seek innovative alternatives related to the detection and quantification of pollution levels in the Chinchiná River by measuring extracellular enzymatic activity with regard to the determination of the enzymatic activity of phosphatase and β-glucosidase on 4-nitrophenylphosphate and 4-nitrophenyl-β-D-glucopyranose, respectively, in the presence of great microorganism activity due to the presence of various levels of contamination and favorable environmental conditions.

II. MATERIALS AND METHODS

A. Study Area

The Chinchiná River originates in the moor of Letras at an altitude of 3600 m and empties into the Cauca River near the Hacienda el Retiro at an altitude of 800 m. It stretches approximately 75 km and covers an estimated area of 113, 264 ha (Fig. 1). The Chinchiná River is a water source for the populations of Manizales, Villamaría, Chinchiná and Palestina. The Chinchiná River Basin has an area of 4,065 km^2, comprises part of the department of Caldas from the moor of Letras, to the village of Arauca in the municipality of Palestina [20].

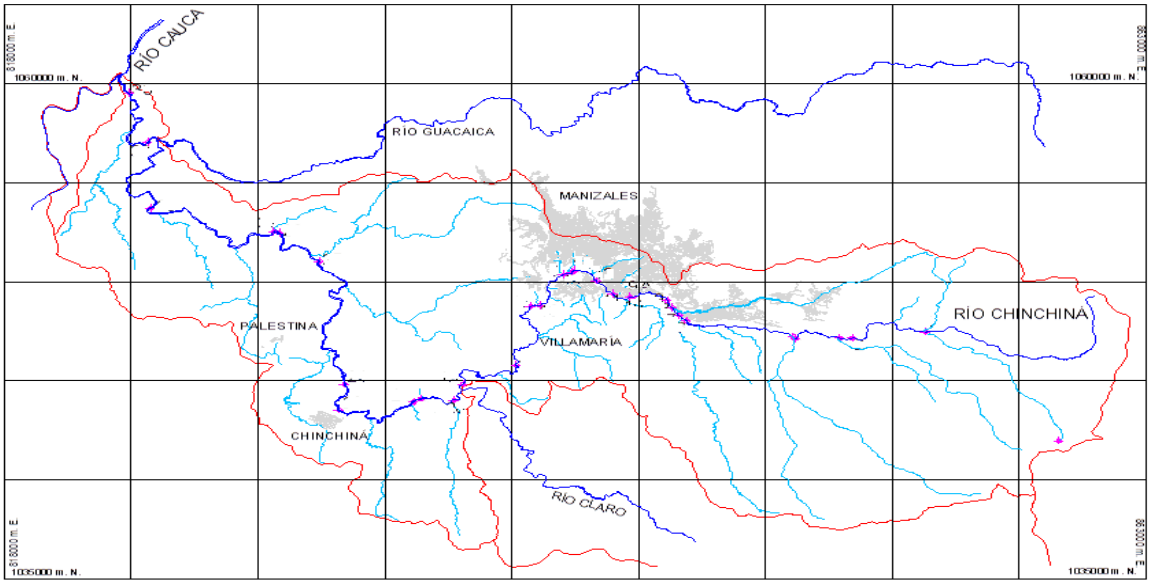

Fig. 1 Chinchiná River route through different populations of the Department of Caldas [20]

The region of the river's location belongs to the hydrographic system of the Cauca River basin on the western side of the Central Cordillera. The Chinchina River can be divided into three distinct areas according to altitude, precipitation distribution, variations in temperature and relative humidity, as described in Table 1.

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TABLE 1 CHINCHINÁ RIVER AREAS

AREA	ALTITUDE (m)	PRECIPITATION (mm/year)	TEMPERATURE (°C)	HUMIDITY (%)
Uptown	3.250 – 2.300	600 – 800	18,4	75 – 80
Middle	2.300 – 1.400	1000 – 2000	22,3	73 – 75
Low	1.400 – 1.840	2500 – 3000	28,6	70 – 75

The Chinchiná River varies throughout the valley depending on the presence of mountains and the predominant type of geographic material. In the upper valley, it is shaped like a narrow and deep "V"; in the middle portion, it presents a wide valley with a flat bottom, partially forming a narrow throat, but then expands again as it becomes filled by alluvial and fluvial-volcanic deposits.

B. Determination of the Sampling Stations

Five sampling stations in the Chinchiná River (Fig. 2) were identified, as contemplated in Resolution No. 079 of March 22, 2007 issued by the General Director of the Corporación Autónoma Regional de Caldas (CORPOCALDAS) amending Resolution No. 046 of February 23, 2007, which established quality objectives of water resources in the basin of Chinchiná River, within the area of jurisdiction of CORPOCALDAS (Table 2).

TABLE 2 SUB-DIVISION INTO FIVE ZONES ACCORDING TO THE POTENTIAL USE

STAGE	LOCATION	TOTAL LENGTH (m)	PREPONDERANT USE
I	Between stations E-1, Finca la Zulia and E-3, after intake Aguas de Manizales, from 0.00 m to 4494.50 m.	4494, 50	Human and domestic consumption
II	Between stations E-3, after intake Aguas de Manizales and E-5, After Quebrada Tolda Fria, from 4494.50 to 6825 m,48 m.	6825,48	Livestock
III	Between stations E-5, after Quebrada Tolda Fria and E-7, Puente Lusitania - Vía Panamericana, from 6825,48 m to 11943,40 m.	11943,40	Recreation; primary contact
IV	Between stations E-7, Puente Lusitania - Panamericana Road and E-20, before Quebrada San Juan, from 11943,40 to 30780,11 m.	30780,11	Energy generation; aesthetic use
V	Between stations E-20 Before Quebrada San Juan and E-30 bridge Hacienda El Retiro, from 30780,11 to 68295,72 m.	68295,72	Energy generation; recreational use; secondary contact

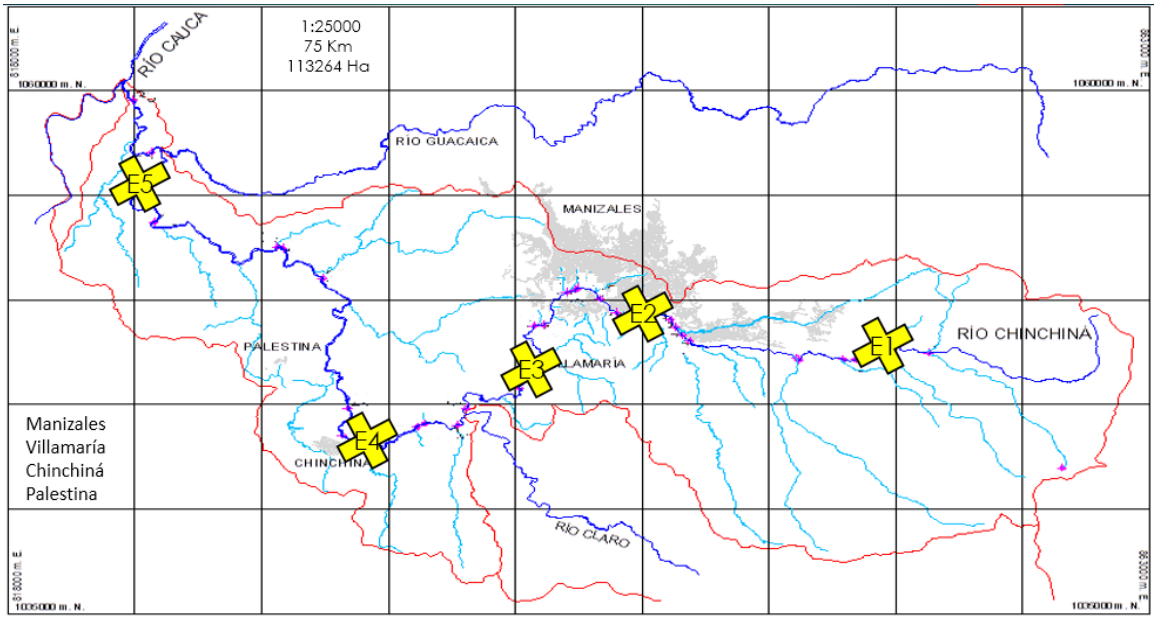

Fig. 2 Location of the sampling stations on the Chinchiná River, modified from CORPOCALDAS - PROAGUA [20]

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C. Field Procedures

At each sampling station, physicochemical parameters were initially determined in situ as listed in Table 3. Sampling was subsequently conducted by collecting spot samples of water and biofilm from five stations on the Chinchiná River, which was preserved in accordance with the type of analysis to be performed. The measurement of physicochemical parameters was conducted performed following notation and according to the methodology proposed in the *Standard Methods for the Examination of Water and Wastewater* [21].

TABLE 3 PHYSICOCHEMICAL PARAMETERS MEASURED IN THE FIELD AS DESCRIBED IN *STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER* [21]

PARAMETER	UNIT	METHOD	SECTION
pH	--	Method 4500-B	Section 4-90
Electrical conductivity	µS/m	Method 2510-B	Section 2-47
Dissolved oxygen (DO) Percentage of oxygen saturation	mg/L - %	Method 4500-O	Section 4500-G
Temperature	ºC	Method 4500-O	Section 4500-G
Turbidity	UNT	Method 2130	Section 2130-B

D. Experimental Procedure

Prior to determining the EEA of β-glucosidase and alkaline phosphatase, a calibration curve was created from a stock solution of 4-Nitrophenol ($O_2NC_6H_4OH$). Dilutions of the stock solution were prepared from a mixture of two parts NaCl solution and one part Na_2CO_3 solution.

The measurement method of EEA consisted of a sample (water and biofilm: epilithon, episammon, periphyton), which were taken to the Laboratory of Environmental Studies in Water and Soil of the University of Caldas within one hour of sample collection. The substrate solution in samples were prepared at a defined concentration (4-Nitrophenyl-β-D-Glucopyranose (conc. > 99 %, M=301.26, Quantity = 1 g., Laboratory: Carl Roth GmbH), and 4-nitrophenylphosphate (conc. > 98%, M=371.12, Quantity=2.5gr. Laboratory: Carl Roth GmbH), colorless) was added. The hydrolysis product of 4-Nitrophenol was detected by spectrophotometry at 405 nm, Nanocolor UV/Vis. Samples were stirred, 5mL/5g were taken from each water or biofilm sample. Each sample was then dissolved in 50 mL sodium chloride (NaCl) solution. Two mL of the resulting suspension was combined with 2 mL of the substrate solution. The sample was then incubated for three hours at 30°C with continuous stirring in a water bath (Model D-20K). After incubation, the reaction was halted by the addition of 2 mL of sodium carbonate (Na_2CO_3) solution. The samples were then centrifuged (Hettich 1205-1; Rotofix 32) at 4500 rpm for 10 minutes at room temperature. A spectrophotometric reading at 405 nm against a blank was then conducted [22]. Alkaline phosphatase (APA) reacts optimally in the range of pH 7.6 to 9.6 [16], and β-glucosidase reacts optimally in pH 6.7 or 7.2 [13] [14]. EEA was measured in vivo, i.e., it operates at the original pH of the water sample.

E. Estimate of the enzymatic extracellular activity

According to Marxsen, et al., [22], extracellular enzymatic activity measured by spectrophotometry can be calculated with the following equation:

$$EEA_x = (Abs_x * D * F)/t$$

EEA_x: Extracellular enzymatic activity of the enzyme x (mol/h)
Abs_x: Absorbance of the final incubation product measured at λ=405nm
D: Dilution factor
F: Photometric factor given by the inverse of the slope calibration curve of 4–nitrophenol (mol/L)
t: Time, in hours

F. Chemical Oxygen Demand (COD)

This procedure was performed according to the closed reflux method (5220 method C. Titulometric Method). Interference determined by the presence of susceptible inorganic substances (which may be oxidized sulfides, sulfites, iodides) were removed by the addition of mercuric sulfate ($HgSO_4$) and silver sulphate (Ag_2SO_4), in order to remove aliphatic straight chain [21].

G. Concentration of Orthophosphates

The determination of orthophosphate in water samples collected from five stations on the Chinchiná River were prepared with NANOCOLOR ortho Phosphate and Total Phosphorus 15 kits (Ref 985080; Test 0.80). This method is based on the

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photometric determination of molybdenum blue after acid hydrolysis and oxidation at 100-120°C at an emission wavelength of 690 nm, and with concentrations ranging from 0.30 to 15.0 mg/LP as PO_4-P and 1.0 to 45.0 mg/L as PO_4^{3-}.

H. Statistical Analysis

Data processing was performed in Statgraphics Plus for Windows, software version 5.1 (DEMO). Shapiro-Wilk normality tests were conducted; analyses of variance (ANOVA) and post hoc Tukey tests ($p < 0.05$) were conducted in order to differentiate between treatments. Kruskal-Wallis tests for data without normal distributions were conducted. Principal Component Analysis (PCA) was conducted in order to determine the associations between EEA and physicochemical control parameters, filtered COD content and orthophosphate concentration. Difference mean and median tests were performed for five seasons, in order to establish statistically significant differences between physicochemical and enzymatic parameters which were analyzed before and after the discharge of pollutants.

I. Determination of Bacterial Counts in Water Samples or Chinchiná River

The presence of *Bacillus* was determined, as potential agents responsible for EEA in the water samples from various seasons on the Chinchiná River, and was determined as follows:

The isolation and culture of bacteria of the genus *Bacillus* was conducted according to the methodology proposed by Madigan, et al. [23]. The method determines a thermal shock (75°C) in order to eliminate other microorganisms (not responsible for extracellular enzyme activity) and to ensure the presence of *Bacillus* species in the samples. Once performed according to the above method, seeding and incubation of the sample on agar PCA (enriched medium) is conducted 72 hours at 37°C. Subsequently, the location and shape of the spore is determined, which proves the presences of *Bacillus* in the water samples collected from the five stations on the Chinchiná River.

III. RESULTS

Table 4 shows changes in the physicochemical control parameters which affect extracellular enzyme activity from different stations and seasons (transition, dry and rainy), collected from the Chinchiná River.

TABLE 4 PHYSICOCHEMICAL PARAMETERS DEFINED BY DIFFERENT SEASONS AND AT DIFFERENT SAMPLING STATIONS

SEASON	TRANSITION				DRY			
PARAMETER / STATION	pH	Electric Conductivity (µS/m)	Turbidity (UNT)	Oxygen Saturation (%)	pH	Electric Conductivity (µS/m)	Turbidity (UNT)	Oxygen Saturation (%)
E1 (Gallinazo Bridge)	7,66	141,00	5,91	77,03	7,27	160,00	6,87	101,35
E2 (Crematorium)	7,13	769,50	65,70	79,22	6,78	350,50	58,07	96,15
E3 (Intake CHEC)	7,93	802,00	51,55	80,98	7,90	1.152,00	59,54	84,20
E4 (Claro River Mouth)	8,21	803,00	77,10	87,28	8,10	1.033,00	84,55	102,15
E5 (Hacienda El Retiro)	8,15	628,00	24,51	92,43	8,45	639,50	37,76	101,85

TABLE 4 CONTINUATION

SEASON	RAINY			
PARAMETER / STATION	pH	Electric Conductivity (µS/m)	Turbidity (UNT)	Oxygen Saturation (%)
E1 (Gallinazo Bridge)	7,58	65,30	47,21	57,90
E2 (Crematorium)	6,99	623,00	35,70	63,10
E3 (Intake CHEC)	7,67	882,50	58,12	85,20
E4 (Claro River Mouth)	7,91	861,50	38,54	95,90
E5 (Hacienda El Retiro)	7,58	691,50	202,19	78,35

Table 5 shows the changes in the filtered Chemical Oxygen Demand (COD) and orthophosphate concentration determined in water samples from the Chinchiná River in the three sampling seasons (transition, dry and rainy). As shown in Table 6, results indicate that orthophosphates concentrations and COD of other Colombian rivers are higher than those reported for the Chinchiná River, as specifically compared to the Medellin River, which presents the highest concentrations of both studied species.

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TABLE 5 DETERMINATION OF CHEMICAL OXYGEN DEMAND (COD) AND ORTHOPHOSPHATE IN DIFFERENT SEASONS AND AT DIFFERENT SAMPLING STATIONS

SEASON	TRANSITION		DRY		RAINY	
PARAMETER / STATION	Orthophosphate (mg/L)	COD (mg/L)	Orthophosphate (mg/L)	COD (mg/L)	Orthophosphate (mg/L)	COD (mg/L)
E1 (Gallinazo Bridge)	0,04	19,29	0,16	17,92	0,25	16,24
E2 (Crematorium)	0,04	4,22	0,14	16,89	0,11	9,28
E3 (Intake CHEC)	0,07	22,09	0,75	41,98	0,65	43,15
E4 (Claro River Mouth)	0,03	26,08	0,07	15,36	0,29	38,51
E5 (Hacienda El Retiro)	0,16	16,32	0,22	10,24	0,63	10,67

TABLE 6 AVERAGE VALUES OF PHOSPHORUS AS PO_4^{3-} IN NEOTROPICAL AQUATIC ECOSYSTEMS [24 - 26]

Types of ecosystems	PO_4^{3-}(mg/L)	COD (mg/L)
Amazon River	0,001	3,00 – 5,00
Tributaries of the Amazon River	0,02	3,45
Medellín	2,00	148,40
Magdalena	0,55	60,00
Cauca	0,07	50,00
San Jorge	0,23	-
Sinú	0,80	-
Streams in the Andean Region	0,001	20,00

According to Table 7, results indicate that EEA for glucosidase and phosphatase in all sampling stations were higher in the dry season than in the rainy season. Results indicate that glucosidase EEA was only observed at one station (E5) during the transition season, and at two stations during the dry season (E2 and E3) while phosphatase EEA was observed during all three sampling periods but not at all stations.

TABLE 7 EXTRACELLULAR ENZYME ACTIVITY REPORT FOR PHOSPHATASE AND B-D-GLUCOSIDASE IN WATER AND BIOFILM SAMPLES IN DIFFERENT SEASONS

	SEASON	TRANSITION		DRY		RAINY	
STATION	SAMPLE	Phosphatase ($EEA/10^{-3}$) (mM/g/h)	Glucosidase ($EEA/10^{-3}$) (mM/g/h)	Phosphatase ($EEA/10^{-3}$) (mM/g/h)	Glucosidase ($EEA/10^{-3}$) (mM/g/h)	Phosphatase ($EEA/10^{-3}$) (mM/g/h)	Glucosidase ($EEA/10^{-3}$) (mM/g/h)
E1 (Gallinazo Bridge)	**Water**	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
		0,0000	0,0000	0,0000	0,0000	0,3025	0,0000
	Biofilm	59,5405	0,0000	431,7319	0,0000	16,9396	9,2512
		0,0000	0,0000	0,0000	5,2180	16,9396	3,9576
E2 (Crematorium)	**Water**	0,9327	0,0000	15,1750	14,4188	0,1765	0,0000
		0,0000	0,0000	42,1472	0,0000	0,4285	0,0000
	Biofilm	46,1805	55,6333	225,1550	33,7027	89,0335	131,3824
		51,9782	34,2068	229,9444	141,3394	327,3721	56,7677
E3 (Intake CHEC)	**Water**	65,9685	0,0000	64,7081	5,3440	0,0000	0,0000
		49,9616	0,4285	59,2885	3,5795	0,4285	0,0000
	Biofilm	53,9528	0,0000	176,6302	150,1621	294,3500	35,9714
		44,6120	0,0000	191,3767	165,9169	117,2661	174,8657
E4 (Claro River Mouth)	**Water**	52,9445	0,0000	0,0000	0,0000	0,0000	0,0000
		51,9362	0,0000	0,0000	0,0000	0,0000	0,0000
	Biofilm	6,2263	0,0000	0,0000	252,8834	18,3260	24,5019
		14,7969	0,0000	261,8321	272,9235	196,1661	0,0000
E5 (Hacienda El Retiro)	**Water**	14,2928	0,0000	0,0000	0,0000	0,0000	0,0000
		12,5282	66,7247	0,0000	0,0000	4,5878	0,0000
	Biofilm	0,0000	0,0000	224,7769	227,4237	40,5087	7,8648
		0,0000	52,3563	211,7949	224,0206	54,8771	31,6860

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A. Statistical Analysis

As shown in Table 8, significant differences were observed in the activity of glucosidase and phosphatase in biofilm samples from all sampling season, as well as for phosphatase activity in water. According to post-hoc Tukey tests, significant differences in catalysis were observed in the phosphatase in biofilm samples, specifically between activity levels present in the transition and dry seasons. Glucosidase activity in biofilm demonstrated significant differences between the rainy and dry between, as well as between the transition season and dry season (Table 9 through Table 12).

TABLE 8 COMPARISON OF ENZYME CATALYSIS BETWEEN SAMPLING SEASONS

EEA	***p*-Valor**	
	Biofilm	**Water**
Glucosidase	0,0006	0,4829
Phosphatase	0,0034	0,0536

TABLE 9 POST HOC TUKEY TEST FOR PHOSPHATASE IN BIOFILM AT VARIOUS SAMPLING STATIONS

Contrast	***Sig.***	***Difference***	***+/- Limits***
18/12/2013 - 25/04/2013		89,4492	110,56
18/12/2013 - 31/07/2013		-78,1463	110,56
25/04/2013 - 31/07/2013	*	-167,595	110,56

* denotes statistical significance.

TABLE 10 POST HOC TUKEY TEST FOR GLUCOSIDASE IN BIOFILM AT VARIOUS SAMPLING STATIONS

Contrast	***Sig.***	***Difference***	***+/- Limits***
18/12/2013 - 25/04/2013		33,4052	77,2097
18/12/2013 - 31/07/2013	*	-99,7342	77,2097
25/04/2013 - 31/07/2013	*	-133,139	77,2097

* denotes statistical significance.

TABLE 11 POST HOC TUKEY TEST FOR PHOSPHATASE IN WATER AT VARIOUS SAMPLING STATIONS

Contrast	***Sig.***	***Difference***	***+/- Limits***
18/12/2013 - 25/04/2013		-24,2641	24,3106
18/12/2013 - 31/07/2013		-17,5395	24,3106
25/04/2013 - 31/07/2013		6,72457	24,3106

* denotes statistical significance.

TABLE 12 POST HOC TUKEY TEST FOR GLUCOSIDASE IN WATER AT VARIOUS SAMPLING STATIONS

Contrast	***Sig.***	***Difference***	***+/- Limits***
18/12/2013 - 25/04/2013		-6,71532	13,827
18/12/2013 - 31/07/2013		-2,33423	13,827
25/04/2013 - 31/07/2013		4,38109	13,827

* denotes statistical significance.

As shown in Fig. 3A and 3B, a greater dispersion of data occurs in the transition season for both water and biofilm samples in regard to glucosidase activity, which further supports the conclusion that the data do not represent average statistics as suggested by the lack of a normal distribution. The data are most homogenous during the dry season, followed by the rainy season.

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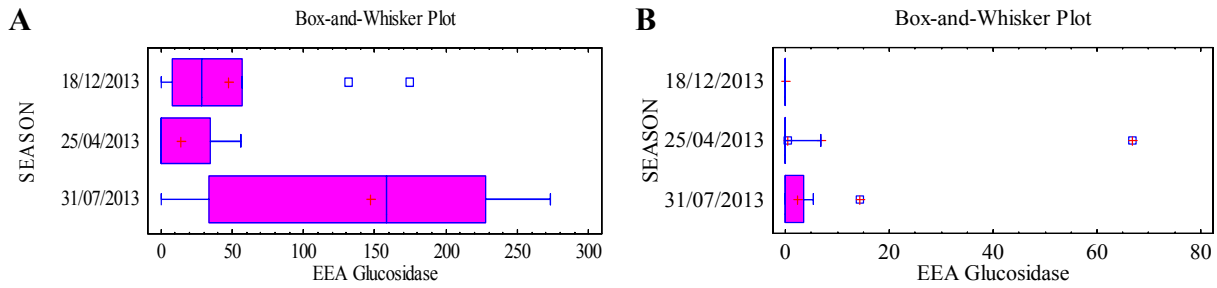

Fig. 3 **A.** Comparison of glucosidase extracellular enzyme activity in biofilm samples **B.** Comparison of glucosidase extracellular enzyme activity in water samples

As shown in Figure 4A, greater uniformity is evident in the dry season followed by the transition season for biofilm samples. It should be noted that in the rainy season, greater dispersion occurs in the data. As shown in Figure 4B, similar behavior is displayed by phosphatase in the transition and dry seasons in water samples, as indicated by the dispersion of data and homogeneity in catalysis represented during the rainy season.

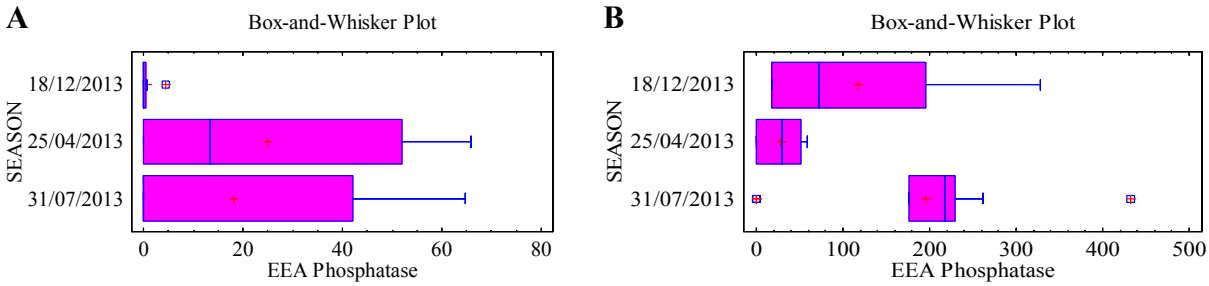

Fig. 4 **A.** Comparison of phosphatase extracellular enzyme activity in biofilm samples **B.** Comparison of phosphatase extracellular enzyme activity in water samples

According to Principal Component Analysis, a correlation exists between the EEA of phosphatase and pH in water samples (Fig. 5A) and between the EEA of glucosidase and temperature in both water and biofilm samples (Fig. 5B and 5C) during the transition season. Figures 6A and 6B illustrate the relationship between the concentration of orthophosphates and COD, respectively, with the EEAs of phosphatase and of glucosidase in water sanokes. In biofilm samples, a relationship was determined between pH and the catalytic activity of phosphatase (Fig. 6C). Figure 7A illustrates the relationship between temperature and the EEA of phosphatase in water samples, which is alternative to the relationship demonstrated between the same items in biofilm samples, in which the catalytic activity of phosphatase was not related to physicochemical control parameters (Fig. 7B).

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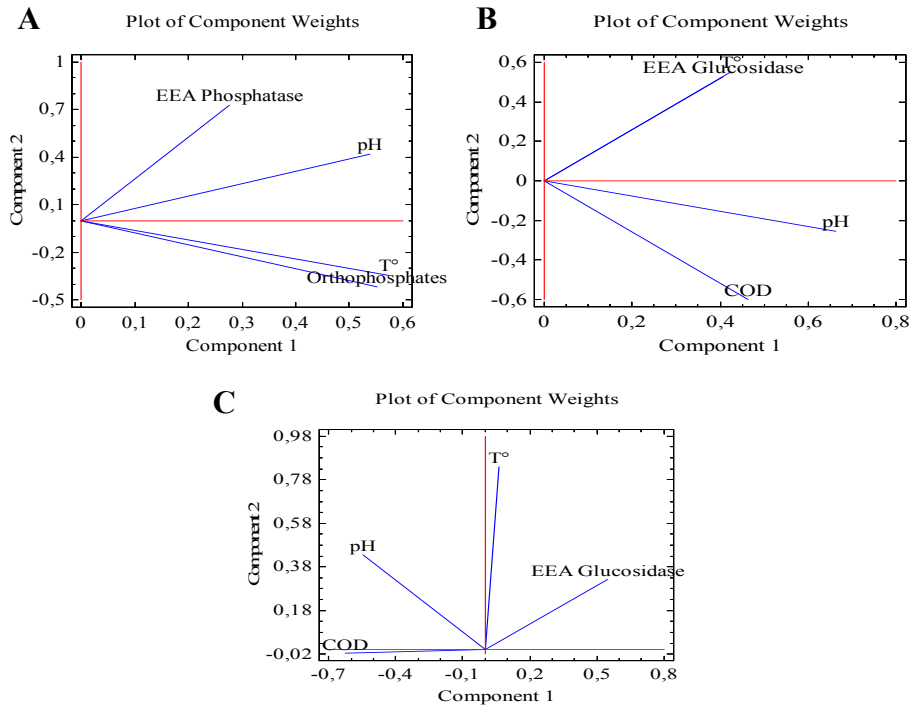

Fig. 5 Principal Component Analysis. **A.** EEA of phosphatase and physicochemical control parameters in water samples **B.** EEA of glucosidase and physicochemical control parameters in water samples **C.** EEA of glucosidase and physicochemical control parameters in biofilm samples during the transition season

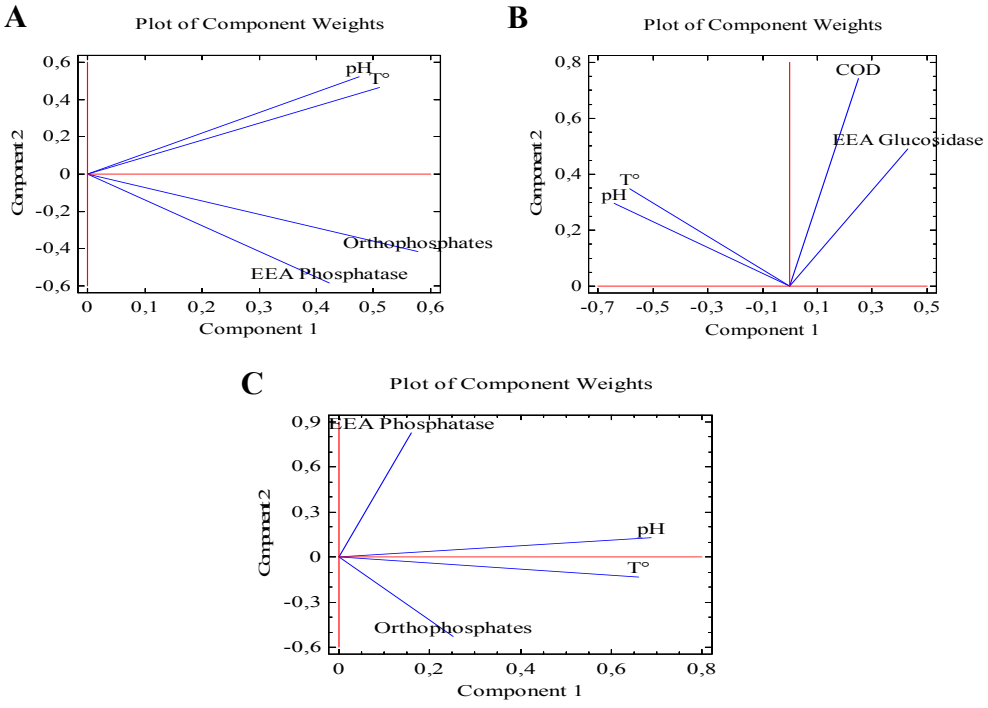

Fig. 6 Principal Component Analysis **A.** EEA of phosphatase and physicochemical control parameters in water samples **B.** EEA of glucosidase and physicochemical control parameters in water samples **C.** EEA of glucosidase and physicochemical control parameters in biofilm samples during the dry season

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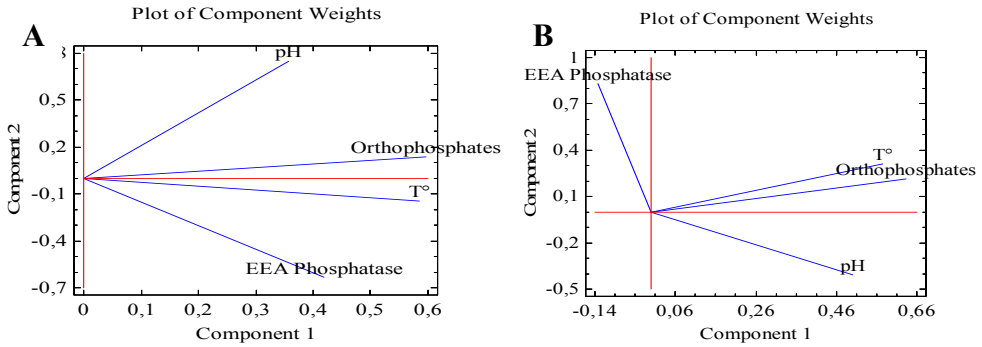

Fig. 7 Principal Component Analysis. **A.** EEA of phosphatase and physicochemical control parameters in water samples **B.** EEA of phosphatase and physicochemical control parameters in biofilm samples during the rainy season

According to PCA results in Fig. 8 and Fig. 9, relationships were identified between EEA of glucosidase and filtered COD, as well as between the EEA of phosphatase and the concentration of orthophosphates in water samples during the dry season.

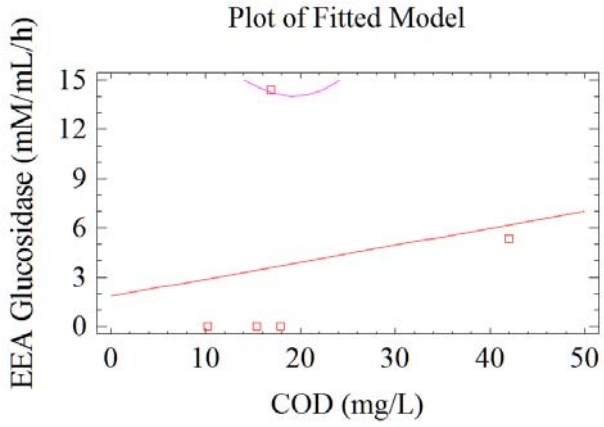

Fig. 8 Relationship between EEA of glucosidase and filtered COD in water samples during the dry season

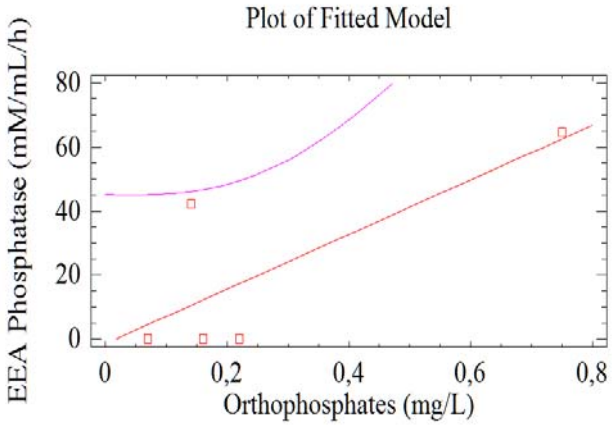

Fig. 9 Relationship between EEA of phosphatase and concentration of orthophosphates in water samples during the dry season

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B. Identification of Bacteria in Water Samples from the Chinchiná River

The successful isolation of bacteria of the genus *Bacillus* was performed, according to the methodology proposed by Madigan, et al. [23]. Such identification was successful for samples from the E2, E3 and E5 stations, which represent the only stations from which samples demonstrated bacterial growth. Incubation was performed for 72 hours, after which the qualitative identification of organisms was achieved through coloration of the colonies. Additionally a gram stain was performed, with red/purple results indicating the presence of gram-positive bacteria. The position of the spore was also identified, as an important parameter according to which the probable *Bacillus* species present in water samples was identified (Fig. 10). Of the samples tested, all showed bacteria spores in a central position, oval or cylindrical in shape and with non-distended sporangium (Table 13).

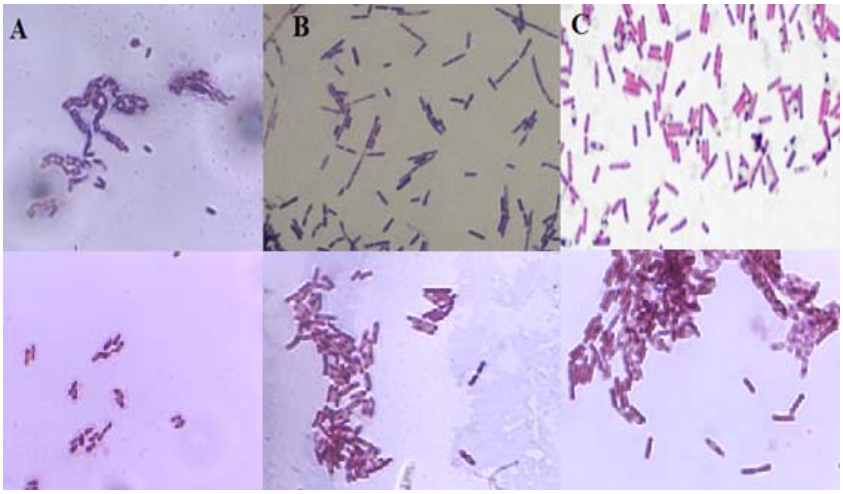

Fig. 10. Identification of *Bacillus* by gram stain. **A.** Station 2 (Crematorium), **B.** Station 3 (Intake CHEC) and **C.** Station 5 (Hacienda El Retiro).

TABLE 13 FEATURES REPRESENTATIVE OF *BACILLUS* SPECIES

Oval or cylindrical spore, aerobic facultative; hydrolyzed casein and starch; nondistended sporangium, thin spore wall.		
Feature	**Species**	**Spore position**
Thermophilic and acidophilic	*B. coagulans*	Central or terminal
	B. acidocaldarius	Terminal
Mesophilic	*B. Licheniformis*	Central
	B. cereus	Central
	B. anthracis	Central
	B. megaterium	Central
	B. subtilis	Central
	B. thuringensis	Central

IV. DISCUSSION

During the three studied seasons, significant differences in EEA of phosphatase and glucosidase (in water and biofilm) was detected due to temperature, thereby indicating a relationship between these two variables. The relationship between pH and EEA changed based on the sampling season. EEA in biofilm and EEA of phosphatase demonstrated no significant differences based on pH during any of the sampling seasons. In water samples, a relationship was found between EEA of phosphatase and pH in both the dry and transition seasons. A relationship between EEA glucosidase and pH in was identified in water samples during the rainy season, and in biofilm samples during the dry season.

According to post hoc Turkey analyses, significant differences in catalysis by phosphatase in biofilm samples were determined between the dry season and the transition season. EEA of glucosidase on biofilm also demonstrated significant differences between the rainy and dry seasons, and between the transition and dry seasons.

During the dry and rainy seasons, EEA of phosphatase and glucosidase was observed in five sampling stations on the Chinchiná River, indicating low availability of food rich in dissolved organic carbon (DOC) and phosphorus. During the rainy season, EEA of glucosidase was not observed in the five sampling stations on the Chinchiná River, indicating availability of COD for the bacteria present in the water.

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According to the notation given by Marxen, et al., [22], (EEA Low: < 20 mM/L; EEA Media: 21 - 80 mM/L; EEA High: > 80 mM/L), low EEA was observed for both glucosidase and phosphatase in water samples and biofilm samples during the three sampling seasons on the Chinchiná River, though that of phosphatase was greater.

According to the results reported by Giraldo, et al., [27] from the Medellin River (Environmental Hall Station), results of the present study are in accordance based on the enzyme activity of phosphatase with respect to glucosidase in all sampling seasons (rainy, transition and dry) in water and biofilm samples.

Glucosidase demonstrated increased activity in the biofilm samples, which is in accordance with results reported by Giraldo, et al., [24], who also concluded that there is increased activity in phosphatase in samples collected from the Medellin River during its rainiest season (December 2010 and February, March and April 2011), and which is similar to results related to the Chinchiná River. Due to the low availability of dissolved organic carbon (DOC) and phosphorus in the water, it was found most enzymatic activity by glucosidase and phosphatase.

EEA increased largely as a result of the dilution effect caused by rainfall during the transition and rainy seasons, and which is consistent with results reported by Cunha, et al., [10] who found that most enzyme activity occurs when the concentration of bioavailable organic matter in the water falls below critical levels (< 0.05 mg/L) [21, 28, 29].

According to the obtained results, the impact of temperature on the enzymatic activity of glucosidase and phosphatase is consistent with results reported by Pohlon, et al., [30] who found that extracellular enzyme activity depends primarily on temperature, in addition to the diversity and richness of microorganisms, specifically bacteria and algae. The presence of *Bacillus* bacteria was observed in water samples from stations E2, E3 and E5, where further contamination by organic matter was observed.

Koch, et al., [31] Zweifel [32], Pomeroy and Wiebe [33] and Loveland, et al., [34] reported the temperature affects the affinity of enzyme systems. These results agree with those obtained in samples from the Chinchiná River, in which it was observed that temperature affects the catalytic activity of both glucosidase and phosphatase. The results regarding the relationship between temperature and enzyme activity in the Chinchiná River agree with those obtained by Patel, et al., [35] who reported the direct relationship between temperature and the enzymatic activity of alkaline phosphatase (APA) and the abundance of microorganisms in the Uranouchi, Japan River.

According to Principal Component Analysis and the resulting trend graphs, a correlation was found between phosphatase and glucosidase EEA in water samples during the dry season as related to the concentration of orthophosphates and filtered COD, respectively. The results indicate that higher concentrations of orthophosphates result in more EEA of phosphatase, and that higher concentrations of COD result in more EEA of glucosidase.

V. CONCLUSIONS

Generally, differences in the EEA between stations during the same period (dry and transition) were observed, due to the bioavailability of nutrients according to the station location. For example, stations E2 (Crematorium) and E3 (Intake Municipal CHEC) have a higher degree of pollution discharge from the municipalities of Manizales and Villamaría, resulting in lower EEA at these stations. These results are contrary to reported EEA at stations E1 (Gallinazo Bridge) and E4 (Rio Claro) which presented higher EEA, because these stations receive wastewater discharge at lower concentrations, emphasizing the decontaminating effect of the river geography.

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