Proteolytic Enzyme Activity and Its Relationship with Physicochemical and Microbiological Indicators in Freshwater Ecosystems of Western Cuba

Katherin Izquierdo Fiallo · Jeny Adina Larrea Murrell ⬝ · Daysi Lugo Moya · Marcia M. Rojas Badía

Abstract The enzymatic reactions of microbial communities are rapid and adequate to changes in the composition of organic matter and environmental factors; therefore, they can be used as descriptors of biological responses in freshwater systems. The objective of this work was to determine the proteolytic enzymatic activity in the Almendares and San Juan rivers (western Cuba) and their relationship with physicochemical and microbiological indicators. The Almendares River (Havana, Cuba) presented water quality index between very poor and unusable for consumption, which makes impossible for recreational uses. In contrast, the waters of the San Juan River (Artemisa, Cuba) are classified as excellent and good in most of the sampling stations during the study period. The greatest proteolytic activities were detected in the San Juan River compared to the Almendares River, which indicates that in more contaminated ecosystems, the proteolytic enzyme activity decreases compared to another less impacted system. In addition, the annual variation in the proteolytic activity of both rivers was evidenced. In the San Juan River, a correlation was observed among proteolytic activity, the concentration of proteolytic bacteria and the physicochemical indicators of water quality, suggesting that proteolytic activity is related to naturally occurring contaminants. In contrast, in the Almendares River, this relationship was not observed, which suggests that the proteolytic activity is not directly affected by the indicators measured in this study.

Keywords Freshwater ecosystems · Water quality index · Proteolytic bacteria · Enzymatic activity

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1 Introduction

Rivers constitute a fundamental source of freshwater available for different uses such as human consumption, recreation, industry, and agriculture. However, at present, most are strongly subject to wastewater discharges of various origins (Franco-Anaya et al. 2016; Afroz and Rahman 2017). The increase of pollutants in the water has caused a decrease in the self-purification capacity of these freshwater ecosystems and the deterioration of their quality (Quiroz et al. 2018). In the coming years, an increase in the demand for water is expected due to the increase of the population, the increase in living standards, and continuous economic development. Meeting this future demand will depend not only on the quantity of water available but also on its quality (Nayar 2020). To determine the water quality in river systems, it is necessary to evaluate physicochemical indicators of contamination such as turbidity, electrical conductivity, salinity, pH, concentration of dissolved oxygen, nitrogen, and phosphorus and microbiological

indicators of fecal contamination such as *Escherichia coli* and enterococci (WHO 2018). From these indicators, it can determine the water quality index that allows summarizing the results of the water quality indicators evaluated in a single value. This value provides an overview of the ecological state of the ecosystem in a given time and place, which allows a better communication of the results to the entities in charge of their management and care (Tirkey et al. 2013).

However, the measurement of physicochemical and microbiological variables only provides information about the main sources of contamination and the concentration of chemical and microbiological contaminants in aquatic environments. These variables do not indicate the impact of pollutants on the composition of the microbial community and its functioning (Kutvirt 2013). Therefore, to determine the ecological status of river systems, it is also necessary to quantify the impact of anthropogenic pressure on the functioning of freshwater ecosystems taking into account their temporal variability (Halliday et al. 2014). Microbial communities are highly sensitive to environmental changes as a result of their rapid life cycle and their ability to use different carbon sources through the production of extracellular enzymes. Enzymatic reactions are rapid and adapted to changes in the composition of organic matter and environmental factors, so they can be used to characterize the functioning of bacterial communities in aquatic ecosystems (Schneider and Topalova 2009; Sabater et al. 2016). Microbial enzymes from aquatic microorganisms include proteases, amylases, and pectinases, among others (Singh et al. 2019).

In Cuba, wastewater treatment is not efficient and urban rivers receive high levels of untreated wastewater that contain a large amount of chemical and biological pollutants. The Almendares River (Havana, Cuba) is one of the main rivers in the west of the country that is greatly affected by urban activities. It receives around 249 polluting sources from different sectors of the economy and urbanization (National Institute of Hydraulic Resources 2010). On the contrary, the San Juan River (Artemisa, Cuba) is located in a rural area in the "Sierra del Rosario" Biosphere Reserve. Its waters have a good ecological status and a good microbiological quality (Larrea et al. 2009). In these freshwater ecosystems, studies have been conducted on the water quality and structure of microbial communities (Larrea et al. 2014; Romeu et al. 2015), as well as in vitro production of extracellular enzymes by strains isolated from

Almendares River (Larrea et al. 2018). However, the enzymatic activity of these ecosystems has not been evaluated and few studies related to this topic exist around the world (Jaramillo et al. 2016; Mullen et al. 2018). Most studies of enzyme activity in aquatic ecosystems have focused primarily on lentic water bodies such as highly polluted lakes and bays (Wang et al. 2017; Li et al. 2019), so little is known about the natural functioning of lotic ecosystems like rivers. The aim of this work was to determine the proteolytic activity in the Almendares and San Juan rivers and their relationship with physicochemical and microbiological indicators.

2 Materials and Methods

2.1 Study Area and Sampling

To analyze the proteolytic enzyme activity at Almendares and San Juan rivers, sampling was done in February, April, and June during the years 2016 and 2017 at three sampling stations in each river. The sampling stations in the Almendares River were Río Cristal (23° 01′ 59.99″ N, 82° 24′ 03.77″ E), Paila (23° 03′ 23.94″ N, 82° 24′ 09.75″ E), and Puente de Hierro (23° 07′ 36.55″ N, 82° 24′ 40.22″ E) (Larrea et al. 2014). In the San Juan River, the sampling stations were Presa El Palmar (22° 50′ 42.35″ N, 82° 56′ 23.33″ E), Presa San Juan (22° 50′ 46.01″ N, 82° 56′ 27.09″ E), and Baños del San Juan (22° 49′ 24.02″ N, 82° 55′ 35.08″ E) (Romeu et al. 2015). The samples were taken in the morning at a distance of 1 m from the shore and 15 cm deep. The samples were transferred to the laboratory in sterile plastic bottles of 500 mL in a refrigerator (4 °C) and were processed in a period of less than 12 h (AFNOR 2009).

2.2 Determination of Physicochemical Indicators

The measurement of physicochemical indicators, such as temperature, pH, electrical conductivity, total dissolved solids, and dissolved oxygen, was carried out in situ with the use of the multimeter (Toledo). The determinations of inorganic nutrients (nitrate, nitrite, ammonium, and phosphate) were performed using colorimetric methods and a spectrophotometer as described APHA (2012).

2.3 Determination of Microbiological Indicators of Fecal Contamination

The concentration of Escherichia coli and enterococci was determined using the membrane filtration technique (FM), through sterile cellulose nitrate membranes (Sartorius, with a pore size of 0.45 μm and 47-mm diameter) on Agar Chromocult (Merck, Darmstadt, Germany) and Agar Chromocult for enterococci (Merck, Darmstadt, Germany), respectively. Dark blue or violet colonies obtained after 24 h of incubation at 37 °C on Agar Chromocult were considered Escherichia coli colonies (Koffi et al. 2014). Red colonies obtained after 24 h of incubation at 37 °C on Agar Chromocul for enterococci were considered enterococci colonies (Koffi et al. 2014). The samples were analyzed in triplicate and in all cases; the results were expressed in colony-forming units (CFU) per 100 mL of sample.

2.4 Spectrophotometric Determination of Chlorophyll a

The spectrophotometric determination of chlorophyll a was carried out using the extraction method with 80% acetone according to the methodology proposed by Jeffrey and Humphrey (1975). Each sample was made in triplicate and the results were expressed in micrograms per liter.

2.5 Determination of the Concentration of Proteolytic Bacteria

From the water samples of both rivers, 1 mL was taken to perform serial dilutions to 10^{-2} and 10^{-3} in the case of samples of San Juan River, and 10^{-4} and 10^{-5} in the case of Almendares Rivers. 100 μL of each dilution was seeded by dissemination in Caso Agar (Merck) and incubated at 37 °C for 48 h. After this time, the count of proteolytic bacteria colonies was performed. The concentration of the proteolytic bacteria in the water sample was expressed in colony-forming units (CFU) per 100 mL of sample. Of all the grown colonies, the most representative in terms of quantity and morphological differences were selected and were isolated in Caso Agar (Merck).

2.6 Micromorphological Characterization of Proteolytic Bacteria

The micromorphological characterization and the bacterial response to the Gram staining were determined by the use of the optical microscope (1000×).

2.7 Determination of Proteolytic Enzyme Activity

For the determination of proteolytic activity, 500 μL of casein (1%), dissolved in a phosphate buffer (pH 6.0, 50 mM) with constant agitation and boiling for 10 min, was added in a test tube with 500 μL of the water sample (diluted 1:10 with phosphate buffer pH 7.0) and incubated at 30 °C for 10 min. To stop the reaction, 1 mL of 0.4 M Trichloro Acetic Acid (TCA) was added with agitation (Pandey et al. 2003). It was centrifuged at 19,100 gravities for 10 min. A control was prepared as control of casein hydrolysis by temperature, in which the previously prepared casein was incubated at 30 °C for 10 min. Then 1 mL of TCA was added. After this, 500 μL of crude enzyme extract (water sample diluted 1:10 with phosphate buffer pH 7.0) was added. Subsequently, 500 μL of the sample supernatant was added in a test tube along with 2.5 mL of sodium carbonate (0.4 N) and 500 μL of Folin's reagent (1:5 in distilled water). It was left to react in a temperature-controlled bath at 30 °C for 30 min. Absorbance at 660 nm was read (Gandolfi and Peralta 2000). This procedure was performed for both the sample and the control. Absorbance was compared to a Tyrosine standard curve. One unit (U) of protease was defined as the amount of enzyme that reduces 1 mmol of casein to tyrosine per minute (Alquicira 2003). The determinations were made in triplicate.

2.8 Water Quality Index Determination

The water quality index was determined using the water quality variables: pH, electrical conductivity, dissolved oxygen, total dissolved solids, concentration of nitrite, nitrate, ammonium, phosphate, enterococci, and Escherichia coli. The water quality index (WQI) was determined according to the methodology proposed by Yisa and Jimoh (2010). The water quality values were reported according to the classification proposed by Yisa and Jimoh (2010).

2.9 Statistical Analysis

The normal distribution and homogeneity of variance of the data were verified using the Kolmogorov-Smirnov and Cochran-Bartlett tests, respectively. The existence of significant differences among the chemical and microbiological indicators and between the proteolytic activity and the concentration of proteolytic bacteria

was determined. For the cases in which the premises were fulfilled, an analysis of variance (ANOVA) of simple classification was performed for a significance level of 1 or 5%, and the Tukey HSD test was subsequently applied. In cases where the premises of normality and homogeneity of variance were not fulfilled, the non-parametric Wald-Wolfowitz streak test was performed ($p < 0.05$). To evaluate the correlation among the physicochemical indicators, the indicators of fecal contamination, proteolytic activity and the concentration of proteolytic bacteria, the Pearson's correlation coefficient (r, parametric method) was calculated for the cases in which the premises were fulfilled. In those cases were the premises were not fulfilled, the Spearman non-parametric linear correlation test was performed. Statistical analyzes were carried out in the Statistica version 8.0 program (StatSoft 2007).

3 Results

3.1 Water Quality of Almendares and San Juan Rivers

The water quality index in the Almendares River (Table 1) presented values above 300 during the entire study period (February 2016–June 2017), which classified the status of water quality as unusable for consumption. During both years, the value of the water quality index correlated positively with the concentration of phosphate and ammonia ($p < 0.01$) (Online Resource 1), and specifically in 2017, it also correlated with the concentration of enterococci and *Escherichia coli*. In contrast, the San Juan River in 2016 (Table 1) presented water quality index values classified as excellent, with

the exception of the Baños del San Juan station in June, which was classified as very poor quality. In the months of February and April 2017, a change in the water quality is evident in the San Juan River (Table 1) because most of the sampling stations were classified as poor or very poor (indices between 100 and 300). The quality index at the Lago San Juan station in April 2017 confirmed a value of 468, which classified the water as unusable for consumption. In this period, only the Lago San Juan station in February 2017 presented waters with excellent quality. On the contrary, in June 2017, the quality of the waters was maintained in all the sampling stations between excellent (< 50) and good quality (50–100). During the entire study period, in this river, the water quality index correlated positively with the phosphate concentration ($p < 0.01$) (Online Resource 1). In 2016, it also positively correlated with the concentration of total dissolved solids ($p < 0.05$) and in 2017 with the nitrite concentration ($p < 0.01$).

3.2 Proteolytic Activity Detected in the Waters of Almendares River

In the Almendares River, the values of the proteolytic activity showed significant differences between the years 2016 and 2017 (Fig. 1(a, b)). In 2016, the proteolytic enzyme activity values ranged from 0 to 0.021 μmol mL^{-1} min^{-1} (Fig. 1(a)). Proteolytic activity values were obtained in Río Cristal sampling station in February and April, reaching the maximum value in February. In Paila and Puente de Hierro sampling stations, proteolytic enzyme activity was obtained in the 3 months of study, reaching the highest values in the month of June and

Table 1 Water quality index determined in Almendares and San Juan rivers during February 2016–June 2017

Sample stations		2016			2017		
		February	April	June	February	April	June
Almendares River stations	Río Cristal (RC)	*470.9*	*500.3*	*1249.7*	*302.7*	*468.7*	*531.2*
	Paila (P)	*1699.1*	*1557.7*	*2047.1*	*3223.6*	*3462*	*2526.4*
	Puente de Hierro (PH)	*1445.7*	*1532.6*	*1294.3*	*1971*	*2210.1*	*1211.3*
San Juan River stations	El Palmar (S1)	9.7	18.1	36.7	112.3	211.7	18
	Lago San Juan (S2)	13.1	13.5	41	24.5	*468*	60.4
	Baños del San Juan (S3)	22.8	29	254.4	165.8	165.8	74

The italicized values represent water quality unsuitable for drinking (> 300), values < 50 represent waters with excellent quality, values between 50 and 100 represent waters with good water quality, values between 100 and 200 represent waters with poor water quality, and values between 200 and 300 represent waters with very poor water quality according to Yisa and Jimoh (2010)
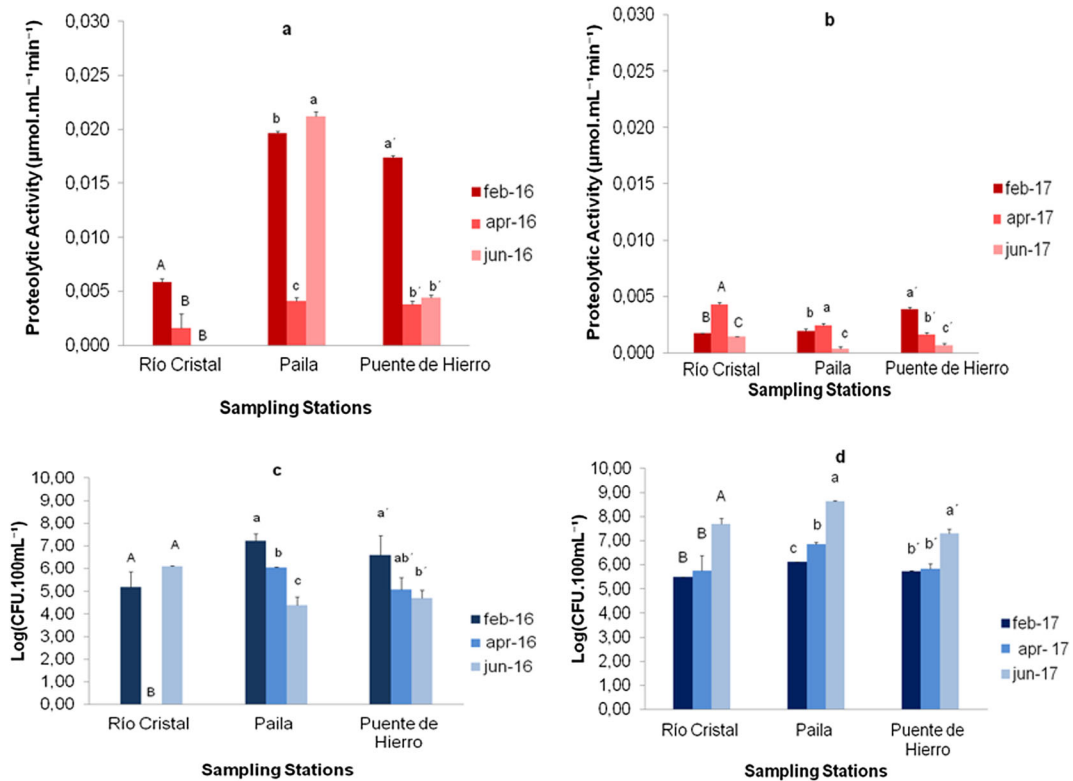
Fig. 1 Values of the proteolytic activity and the logarithm of the concentrations of proteolytic bacteria in the stations of the Almendares River from February 2016 to June 2017. Enzymatic activity in 2016 (A) and in 2017 (B). The logarithm of the concentrations of proteolytic bacteria in 2016 (C) and in 2017 (D). Error bars indicate the standard deviation of $n = 3$. The different letters on the bars (A, B, C; a, b, c; and a′, b′, c′) indicate significant differences between the sampling months in each station according to the ANOVA test (simple)-Tukey HSD ($p < 0.05$)

February, respectively ($p < 0.05$). In 2017, the enzyme activity values ranged from 0.0003 to 0.0042 µmol mL^{-1} min^{-1} (Fig. 1(b)). In Río Cristal and Paila sampling stations the highest activity values were reached in April ($p < 0.05$); however, in Puente de Hierro station, the highest value was reached in the month of February. During the study period (February 2016–June 2017), high concentrations of proteolytic bacteria were detected in the three sampling stations (Fig. 1(c, d)), except for Río Cristal station in April 2016, in which it was not detected. In 2016 (Fig. 1(c)), the concentration of proteolytic bacteria showed logarithmic values between 4.40 and 7.23. In Paila and Puente de Hierro stations, the maximum concentration of proteolytic bacteria was reached in February. In Río Cristal station, the highest concentration of proteolytic bacteria was reached in the months of February and June ($p < 0.05$). In 2017, the logarithmic values were between 5.49 and 8.63 (Fig. 1(d)), with the highest concentration of proteolytic bacteria in the month of June at the three sampling stations ($p < 0.05$). The morphotintorial characteristics of the isolated proteolytic bacteria showed that most of them were Gram-positive rod-shaped endospore-forming (Online Resource 2). The rest of the isolated proteolytic bacteria were Gram-positive rod-shaped non-sporulating, Gram-positive cocci, Gram-negative rod-shaped, and Gram-negative cocci.

Table 2 shows the Pearson's correlation coefficients among proteolytic activity, the concentration of proteolytic bacteria, and the physicochemical and microbiological indicators during the period February 2016–June 2017. As observed, there was no correlation between proteolytic activity or the concentration of proteolytic bacteria with physicochemical and microbiological indicators, but it shows a negative correlation between the concentration of proteolytic bacteria and proteolytic activity, which indicates that increasing or decreasing

Table 2 Pearson's correlations[1] ($p < 0.05$) among proteolytic activity, the concentration of proteolytic bacteria, and the physicochemical and microbiological indicators determined in Almendares River in the period February 2016–June 2017

	Temp.	pH	CE	OD	STD	Sal.	NO_2^--N	NO_3^--N	PO_4^{3-}-P	NH_4^+-N	Clor-α	Ent.	E. coli	Prot. Actv.	Conc. Prot.
Prot. Actv. 2016	−0.318	−0.348	0.014	−0.027	0.015	−0.019	−0.221	0.381	0.565	0.382	−0.224	−0.296	−0.053	1	
Conc. Prot. 2016	−0.061	−0.225	0.074	0.157	0.056	0.080	0.028	0.459	0.590	0.300	−0.629	−0.282	0.330	0.377	1
Prot. Actv. 2017	−0.474	0.449	0.073	0.137	0.061	0.058	−0.072	0.195	−0.127	−0.343	0.548	−0.127	−0.216	1	
Conc. Prot. 2017	0.488	−0.548	−0.2195	−0.428	−0.209	−0.251	−0.240	−0.608	0.191	0.335	−0.274	0.117	0.235	−0.676*	1

Temp., temperature; *CE*, electrical conductivity; *OD*, dissolved oxygen; *STD*, total dissolved solids; *Sal.*, salinity; NO_2^- -*N*, nitrite; NO_3^- -*N*, nitrate; NH_4^+ -*N*, ammonium; PO_4^{3-} -*P*, phosphate; *Ent.*, enterococci; *E. coli, Escherichia coli*; *Prot Actv.*, proteolytic activity; *Conc. Prot.*, concentration of proteolytic bacteria

Positive correlations are indicated in italics

[1] Pearson correlation was performed for all values except those corresponding to the chlorophyll *a* in 2017, where simple linear Spearman correlation was performed

the concentration of proteolytic bacteria decreases or increases protease activity.

3.3 Proteolytic Activity Detected in the Waters of San Juan River

In the San Juan River, the values of proteolytic activity showed differences between the years 2016 and 2017 (Fig. 2(a, b)). In 2016, the enzyme activity values ranged from 0 to 0.109 µmol mL^{-1} min^{-1} (Fig. 2(a)). At the Lago El Palmar station, proteolytic activity was only detected in June, reaching a value of 0.015 µmol mL^{-1} min^{-1}. In the Lago San Juan station, proteolytic activity was obtained throughout the period February–June 2016, reaching its maximum value in April. On the other hand, in the Baños del San Juan station, proteolytic activity was only detected in April, which reached a value of 0.084 µmol mL^{-1} min^{-1}. In 2017, the proteolytic enzyme activity values ranged from 0 to 0.005 µmol mL^{-1} min^{-1} (Fig. 2(b)). Proteolytic activity was not obtained at Lago El Palmar station in February and at Baños del San Juan station in February and April. The highest proteolytic enzyme activity in 2017 was detected at Lago El Palmar station in June and at Lago El San Juan station in February ($p < 0.05$).

During the period, there were differences between the concentrations of proteolytic bacteria detected in 2016 and 2017 (Fig. 2(c, d)). In 2016, the logarithm of the concentration of proteolytic bacteria showed values between 0 and 6.23 (Fig. 2(c)), reaching the highest values in February in each sampling station. No concentrations of proteolytic bacteria were detected in April at the three sampling stations and in June at Lago El Palmar station. In contrast, in 2017 (Fig. 2(d)), concentrations of proteolytic bacteria were detected in all the stations analyzed from February to June; with logarithmic values ranging from 3.32 to 6.91. Unlike in 2016, in 2017, the highest logarithmic concentrations of proteolytic bacteria were obtained in April at each sampling station.

The morphotintorial characteristics determined for the isolated proteolytic bacteria (Online Resource 3) showed that most of them were Gram-positive rod-shaped endospore-forming. The rest of the isolated proteolytic bacteria were Gram-positive rod-shaped non-sporulating, Gram-positive cocci, Gram-negative rod-shaped, and Gram-negative cocci.

Table 3 shows Pearson's correlation coefficients among proteolytic activity, the concentration of proteolytic bacteria, and the physicochemical and

microbiological indicators during the period February 2016–June 2017. In 2016, there was a positive correlation between proteolytic activity and nitrite concentration in the San Juan River, which implies that increasing or decreasing the nitrite concentration increases or decreases the proteolytic enzymatic activity. A negative correlation was also obtained between the concentration of proteolytic bacteria and the nitrate concentration, which indicates that with increasing or decreasing nitrate concentration, the concentration of proteolytic bacteria present in the ecosystem decreases or increases. In 2017, a negative correlation was observed between the concentration of proteolytic bacteria and electrical conductivity, salinity, and the concentration of total dissolved solids, which indicates that the increase or decrease in conductivity, salinity, and concentration of total dissolved solids decreases or increases the concentration of proteolytic bacteria in the waters of the San Juan River. In addition, a positive correlation was obtained between proteolytic activity and chlorophyll *a* concentration.

3.4 Comparison of the Proteolytic Activity Detected in the Waters of Almendares and San Juan Rivers

As seen in Fig. 3(a), the annual average of proteolytic enzyme activity in 2016 was significantly higher in the San Juan River compared to the Almendares River. In 2017, no differences were detected between the annual averages of the proteolytic activities of both rivers. In addition, the annual mean of proteolytic enzyme activity in both rivers was higher in 2016 compared to 2017. Regarding the annual means of the logarithms of the concentration of proteolytic bacteria (Fig. 3(b)), they were higher in the Almendares River regarding the San Juan River in both years of study. Contrary to proteolytic activity, the annual means of the logarithms of the concentration of proteolytic bacteria were higher in 2017 compared to 2016 in both ecosystems.

4 Discussion

4.1 Water Quality Index in Almendares and San Juan Rivers

The water quality index allows summarizing the information of most of the quality indicators to a simple expression, so that they can be easily transmitted to the entities in charge of the care, preservation, and sanitation of aquatic ecosystems (Samboni et al. 2007; Yisa and Jimoh 2010). Table 1 shows how the water quality indices in Almendares River indicate the poor quality of the water during the 2 years of sampling. This is due to the high values of electrical conductivity, salinity, total dissolved solids, ammonia, phosphate, and microbiological indicators of fecal contamination (Online Resource 4). The high concentrations of nutrients found in the Almendares River throughout the study period, and its positive correlation with the resulting water quality indices for both years, indicate that the river receives constant discharges of wastewater containing fecal organic matter. Romeu et al. (2015) warn in the vicinity of the Paila stream the presence of corrals for animal husbandry (e.g., birds, rams, and pigs) and the dumping of raw residues from houses that are not connected to the sewerage and from a pharmaceutical laboratory. All of these polluting sources contribute to making this sampling station the most polluted in the river. Furthermore, this result is in correspondence with that obtained by Arpajón et al. (2011) who stated that the Paila station had the highest values of chemical contamination. The Puente de Hierro sampling station also presented a high concentration of nutrients with respect to the Río Cristal station which is reflected through the water quality index (Table 1). This station corresponds to the estuary area, where there is an accumulation of all the pollutants that are discharged along the basin and being carried out by the current of the river to its mouth.

In the case of the San Juan River, a positive correlation was observed between the phosphate concentration and the water quality index obtained throughout the study period (Online Resource 1). During the samplings carried out during 2016, greenish water was observed, with the abundant flow, so the increase in phosphate concentration could be caused by the resuspension of the nutrients found in the sediments due to turbulence caused by the strong movement of the water. Precisely, in this year, the water quality index also correlated positively with the concentration of total dissolved solids. The concentration of total dissolved solids is indicative of dissolved organic and inorganic matter in a water sample with a diameter less than two micrometers (Roy et al. 2014; Tanor et al. 2014). This can come from a wide variety of human activities such as the use of fertilizers in agriculture, as
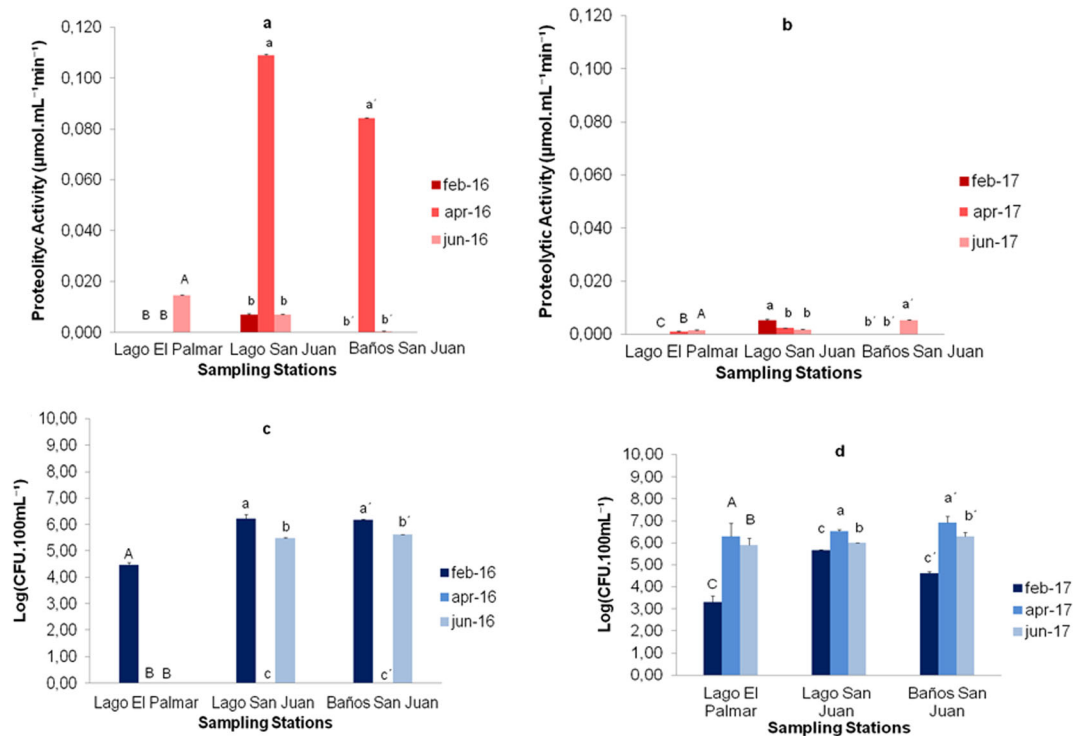
Fig. 2 Values of the proteolytic activity and the logarithm of the concentrations of proteolytic bacteria in the stations of the San Juan River from February 2016 to June 2017. Enzymatic activity in 2016 (A) and in 2017 (B). Logarithm of the concentrations of proteolytic bacteria in 2016 (C) and in 2017 (D). Error bars indicate the standard deviation of $n = 3$. The different letters on the bars (A, B, C; a, b, c; and a′, b′, c′) indicate significant differences between the sampling months in each station according to the ANOVA test (simple)-Tukey HSD ($p < 0.05$)

well as the discharge of wastewater (Tanor et al. 2014). However, high concentrations are not necessarily indicative of contamination. Normally, in rivers, processes of dissolution and accumulation of high concentrations of ions from minerals from rocks and soils occur. Dissolved solids are very important for aquatic life keeping the cell density balanced. Despite this situation, the quality of the waters of the San Juan River in 2016 and in the month of June 2017 was classified as excellent and good (Table 1). In the case of June 2016 and April 2017 at the Baños del San Juan and Lago San Juan sampling stations, the quality of its waters shows that the index detected the variations produced in the values of the physicochemical and microbiological indicators, such as the increase in electrical conductivity, the concentrations of total dissolved solids, enterococci, and *Escherichia coli* (Online Resource 5). In the months of February and April of year 2017, in the San Juan River, an increase in the index was evidenced in most of the sampling stations,

which indicates the decrease in its quality. Drought and increased temperatures can cause the concentration of nutrients in the water column (Roy et al. 2014). The months of February and April correspond to the dry season in Cuba. Artemisa Province (western Cuba) was one of the areas with the greatest deficit in accumulated rainfall according to the spatial analysis carried out by the meteorological institute (INSMET 2018). Also, it was detected an increase of 1.4 °C in the average temperature in February 2017 according to the historical average for the month of February (INSMET 2018), which could have caused the accumulation of nutrients in the water column. These results it is according to the obtained for Ilie et al. (2017), in the Danubio River, Rumanía.

These results demonstrate how the water quality index with a single value, manages to show the spatio-temporal variations in water quality and allows detecting integrated trends in quality changes due to external factors, which converts the index of water quality into

Table 3 Pearson's correlations[1] ($p < 0.05$) among proteolytic activity, the concentration of proteolytic bacteria and the physicochemical and microbiological indicators determined in San Juan River in the period February 2016–June 2017

	Temp.	pH	CE	OD	STD	Sal.	NO_2^--N	NO_3^--N	PO_4^{3-}-P	NH_4^+-N	Clor-α	Ent.	E. coli	Prot. Actv.	Conc. Prot.
Prot. Actv. 2016	−0.139	0.024	0.659	0.169	0.364	0.669	*0.743**	0.505	−0.209	0.152	−0.241	0.357	0.438	1	
Conc. Prot. 2016	−0.460	−0.144	−0.262	0.259	−0.108	−0.206	−0.536	*−0.745**	0.307	−0.248	0.483	−0.145	−0.453	−0.526	1
Prot. Actv. 2017	0.196	−0.263	−0.090	−0.022	−0.083	−0.175	−0.084	0.222	−0.335	0.146	*0.815***	0.088	0.075	1	
Conc. Prot. 2017	0.622	−0.098	*−0.746**	−0.543	*−0.743**	*−0.878***	−0.045	0.012	0.201	0.361	0.006	0.425	0.138	0.324	1

Temp., temperature; *CE*, electrical conductivity; *OD*, dissolved oxygen; *STD*, total dissolved solids; *Sal.*, salinity; NO_2^--N, nitrite; NO_3^--N, nitrate; NH_4^+-N, ammonium; PO_4^{3-}-P, phosphate; *Ent.*, enterococci; *E. coli*, *Escherichia coli*; *Prot Actv.*, proteolytic activity; *Conc. Prot.*, concentration of proteolytic bacteria

Positive correlations are indicated in italics

[1] Pearson correlation was performed for all values except those corresponding to the nitrite in 2016, where simple linear Spearman correlation was performed

one of the most effective tools for the management and evaluation of the quality of surface water.

4.2 Proteolytic Activity Detected in the Waters of Almendares and San Juan Rivers

Proteins constitute the largest component of the biomass of many aquatic organisms, which is why bacterial proteases are considered the main degraders of nitrogenous organic matter in natural aquatic environments (Li et al. 2017). As can be seen in Fig. 3(a), there are significant differences between the annual average of the proteolytic activity detected in 2016 and 2017 in each river. According to INSMET (2018), the year 2017 presented a greater shortage of rainfall than the year 2016. In 2016, a higher activity of proteases was evidenced in each river compared to 2017, which suggests that in less dry periods, enzymatic activity increases as a result of precipitation. Rainfall can cause the dilution of nutrients, which may become less available to the microbial community and, therefore, could increase the production of extracellular enzymes to obtain nutrients from the available organic matter. On the contrary, in drier periods, there is a greater accumulation of nutrients, which may be used by the heterotrophic community as a source of carbon and energy, so they do not waste energy on the unnecessary production of extracellular enzymes as a form of cellular economy. These differences indicate the annual variation in proteolytic enzyme activity. These results agree with those obtained by Giraldo et al. (2014), which obtained the highest activities of the enzymes β-glucosidase and alkaline phosphatase in the rainy period in the Aburra River, Medellín, Colombia.

In addition, significant differences were obtained between the proteolytic enzyme activity in the Almendares River and the San Juan River in 2016. The lowest activity was detected in the Almendares River (Fig. 3(a)), which indicates that in polluted ecosystems the enzymatic activity decreases compared to another less impacted system. These results agree with those obtained by Jaramillo et al. (2016), who obtained the lowest enzymatic activities in sampling stations that presented a high degree of contamination in Colombia.

The seasonal dynamics of extracellular enzymes are not only caused by variations in temperature and bacterial abundance but are also strongly related to the availability of substrates (Tiquia 2011). Enzymes produced by heterotrophic microorganisms have been reported to
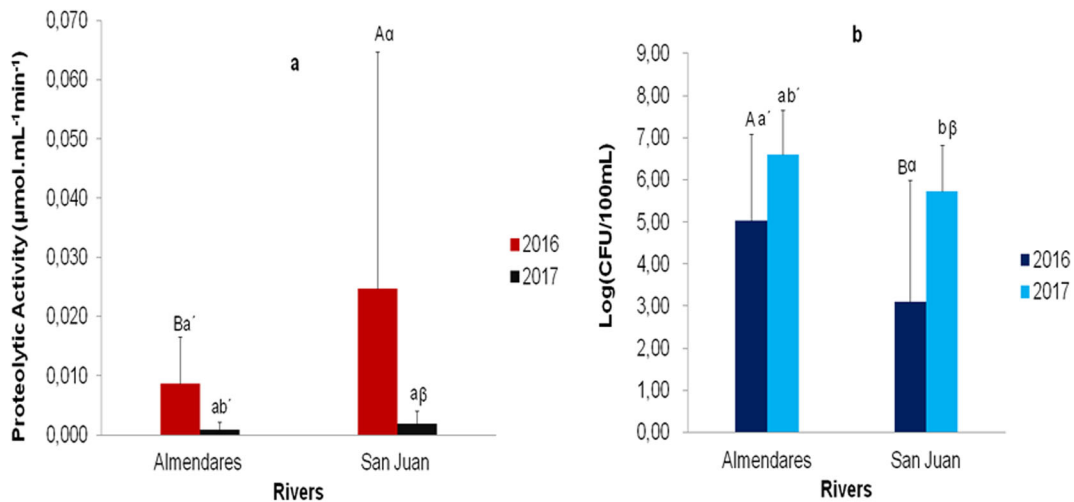
Fig. 3 Annual mean values of the proteolytic activity (A) and annual mean values of the logarithm concentrations of proteolytic bacteria (B) in Almendares and San Juan rivers during 2016 and 2017. Error bars indicate the standard deviation of $n = 9$. The ANOVA (simple)-Tukey HSD tests were made ($p < 0.05$) for proteolytic activity between Almendares and San Juan Rivers during 2017, the logarithm concentrations of proteolytic bacteria in the Almendares River (2016–2017), San Juan River (2016– 2017) and between Almendares and San Juan Rivers during 2017. For the rest of the comparisons, a Wald-Wolfowitz non-parametric test of streaks was made. The different letters on the bars indicate significant differences between the proteolityc activity and the logarithm of the concentrations of proteolytic bacteria between the Almendares and San Juan rivers in 2016 (A and B) and in 2017 (a and b); between 2016 and 2017 in Almendares River (a′ and b′), and between 2016 and 2017 in San Juan River (α and β)

modify their speed in response to different sources of organic matter, affecting the functionality of the ecosystem (Young 2008; Malaver et al. 2014). Substrate utilization capacity in the microbial community changes every year, where some substrates are always used and others only occasionally, depending on the specialized characteristics of the microbial populations (Traving et al. 2016). Therefore, the high enzymatic activities detected in February and June 2016 at the Paila station, in February 2016 at the Puente de Hierro station (both in Almendares River), and in the month of April 2016 at Lago El Palmar and Baños del San Juan stations (San Juan River) may be due to the presence of high concentrations of organic matter. In the case of the Almendares River, the Paila and Puente de Hierro stations are most impacted by anthropogenic discharges in which organic matter is included, and as long as there is a greater amount of biodegradable substances, there is greater enzymatic activity to hydrolyze it. These results are in agreement with those obtained by Tiquia (2011) in the Rouge River, in which it obtained high concentrations of extracellular enzymes at the sampling site corresponding to the highest concentrations of dissolved organic carbon from anthropogenic sources. In the case of the maximums of enzymatic activity observed in the San Juan River stations in April 2016, it may be due to

the accumulation of organic matter produced by low rainfall (INSMET 2018). During the samplings carried out in April at the Lago San Juan and Baños del San Juan stations, little flow was found, including cloudy and greenish water at the Lago San Juan sampling station, suggesting a concentration of nutrients. In addition, another factor that may have contributed to the increase in organic matter in these sampling stations is the resuspension of nutrients found in the sediments by the wind (Sosa-Avalos et al. 2013). This process could have occurred at the Lago San Juan sampling station during the month of April, when there was a lot of wind. In addition, these sampling stations are located near population settlements such as Las Terrazas, Cayajabos, Mango Bonito, La Flora, and Soroa (Arpajón et al. 2012) that can directly or indirectly interact with the river waters and contribute with organic matter to its waters. In the Baños del San Juan station, there is a recreational center in which visitors can produce the resuspension of sediments when bathing, contributing with organic matter to this station. It should be noted that between the Lago San Juan and Baños del San Juan sampling stations is the anaerobic/aerobic oxidation lagoon that has presented breaks throughout the study period and receives volumes of domestic wastewater greater than its retention capacity. These discharges

reach the Baños del San Juan station that is downstream. This situation was observed during the sampling in April 2016. The accumulation of organic matter in these stations can cause an increase in enzymatic activity in the waters resulting from the production of extracellular enzymes by heterotrophic microorganisms, which could explain the peaks of proteolytic activity in April 2016 at the Lago San Juan and Baños del San Juan stations (Fig. 2(a)).

When comparing the results of protease activity and the concentration of proteolytic bacteria (Fig. 1), it was observed that even when proteolytic microorganisms are detected, enzymatic activity is not always detected or vice versa. In April 2016, enzymatic activity was detected at the Río Cristal, Lago San Juan, and Baños del San Juan sampling stations without obtaining concentrations of proteolytic bacteria. This could be due to the fact that, in this study, the total proteolytic activity in the water column was determined; however, in the method to determine the concentration of proteolytic microorganisms, non-cultivable microorganisms, fungal species, and protozoa can also produce extracellular proteases (Jaramillo et al. 2016). Aquatic environments are extremely complex and diverse; in them, there is a great diversity of microorganisms that produce proteases, where many are extracellular, especially in tropical and temperate regions that contain a large amount of organic matter (Zhang et al. 2015). In the Almendares River, the presence of species belonging to the *Enterobacter*, *Citrobacter*, *Enterococus*, *Pseudomonas*, and *Staphylococcus* genders has been demonstrated (Romeu et al. 2011) in which protease-producing species are known to exist. Furthermore, it has been shown that species of filamentous fungi such as *Conidiobolus* spp., *Aspergillus* spp., and *Neurospora* spp., as well as some yeast species, can also produce serine proteases (Ward et al. 2009).

In the month of June 2016 at the Río Cristal (Almendares River) and Baños del San Juan (San Juan River) stations; in the month of February at the Lago El Palmar and Baños del San Juan sampling stations in the years 2016 and 2017; and in the month of April 2017 in the Baños del San Juan station, the presence of proteolytic bacteria was determined; however, no enzymatic activity was obtained. The dynamics of the composition of bacterial communities in aquatic ecosystems and their functions are governed by the availability of dissolved organic matter and inorganic nitrogen sources (Traving et al. 2016), so this situation may be due to various factors; for example, proteases may not being excreted since the producing microorganisms are using other nutrients of the organic matter like lipids or simpler carbon sources that come from non-native sources of contamination (Cunha et al. 2010; Kalwasińska and Brzezinsk 2013; Giraldo et al. 2014). The ability of heterotrophic communities to use different organic sources depends on the enzymes they have and the substrates that are available in the environment (Tiquia 2011). Li et al. (2017) demonstrate that a single bacterial strain of *Bacillus* spp. has the potential to produce high concentrations of proteases in media with casein, gelatin, and elastin, where the type of substrate to which they have access in the water column determines the type of enzyme they produce and if the media does not contain casein (which was the substrate used in this study), this protease is not produced.

Another cause of the existence of proteolytic microorganisms without detecting their proteolytic activity in the Almendares River could be that it is inhibited by conditions such as the concentration of nutrients, humic matter, pH, heavy metals, and salinity. Many of these conditions were measured through physicochemical indicators. In the Almendares River, the presence of high concentrations of heavy metals such as iron and zinc has been demonstrated (Díaz et al. 2019). These heavy metals can affect the enzymatic activity, as well as the growth of the proteolytic microorganisms that produce them (Wei et al. 2010; Huang et al. 2015). Proteases are strongly affected by these contaminants, since they can inhibit their activity by reacting with the enzyme-substrate complex, with the substrate or combining with the protease active group, inhibiting or changing their function. Many proteases possess sulfhydryl groups as catalytic sites or as part of their structure, maintaining the correct conformation of the protein (Wei et al. 2010; Huang et al. 2015). Exogenous lead can destroy the structure of these disulfuric bridges or cover them by inhibiting their activity (Huang et al. 2015). Studies carried out by Wei et al. (2010) demonstrate that high concentrations of zinc inhibit aminopeptidase activity. In addition, metals can inhibit the growth of proteolytic microorganisms, decreasing enzyme production and consequently their activity, due to the toxic effect produced by the inhibition of DNA repair mechanisms, oxidative damage, or the stimulation of apoptosis signals (Huang et al. 2015). However, in this case, low concentrations of proteolytic bacteria were not obtained, which could be due to the defense mechanisms that

some microorganisms have developed to heavy metals, which are based on the accumulation inside them, forming metal-protein associations or blocking its entry through the cell wall or the membrane transport system (Huang et al. 2015). Martínez et al. (2010) demonstrated the presence of several strains resistant to heavy metals like lead, cadmium, and chromium in the Almendares River.

Furthermore, in this river, there is a constant inflow of wastewater that contains abundant amounts of enteric microorganisms such as enterococci and *Escherichia coli* that can produce proteolytic enzymes. This could be related to the negative correlation obtained between proteolytic activity and the concentration of proteolytic bacteria in the Almendares River in 2017 (Table 2). Abundant concentrations of inorganic nitrogenous nutrients and organic matter are also poured into the wastewater along with enteric microorganisms. With the increase in inorganic nutrients, proteolytic microorganisms do not have to produce proteases to obtain nutrients.

In the Lago El Palmar sampling station in April 2016, no enzyme activity was detected, nor was the presence of proteolytic microorganisms. In April 2016, there was low rainfall, which could cause the sedimentation of microorganisms and substances such as organic matter and proteases that were in the water column. This sedimentation could have been the cause of the non-detection of proteolytic activity or of the protease-producing bacteria. According to Schneider and Topalova (2009), the amount of heterotrophic bacteria and organic matter is greater in the sediments than in the water column, for which reason most of the degradation processes occur in the sediments. If we compare the annual average of the enzymatic activity of the Almendares River and San Juan River with the average concentration of proteolytic bacteria, we can see that there are notable differences. The highest concentrations of proteolytic bacteria were obtained in 2017 in both rivers; however, it does not agree with the results of proteolytic activity since the highest average protease activities were obtained in 2016. The year 2017 presented the greatest shortage in rainfall compared to 2016 (INSMET 2018), which could cause a higher concentration of nutrients, making the production of proteases unnecessary. On the contrary, in 2016, despite there being a lower concentration of proteolytic bacteria compared to 2017, there was a higher significant enzymatic activity, which indicates that not only does rainfall favor

the production of extracellular enzymes by proteolytic bacteria but proteolytic activity also depends on other microorganisms that produce extracellular proteases and that may favor the increase in proteolytic enzyme activity detected in this study.

Proteases play an important role in the processes of decomposition and nitrogen acquisition by protein hydrolysis. Its activity generally indicates deficiencies in inorganic nitrogenous sources, since protein hydrolysis allows the primary obtaining of ammonium by deamination of amino acids from proteins (Tsuboi et al. 2014; Traving et al. 2016). The presence of enzymatic activity in most of the sampling stations in both rivers could contribute to the high concentrations of ammonia with respect to other inorganic nitrogenous nutrients found in both rivers during the study period, especially in the Almendares River. In addition, during 2017, a decrease in the enzymatic activity was evidenced in both rivers compared to 2016 due to low rainfall. This situation is reflected in the negative correlation obtained between proteolytic activity and salinity, electrical conductivity, and total dissolved solids in the San Juan River in 2017 (Table 3), which indicates that with the increase of inorganic nutrients decreases enzyme activity.

On the other hand, the proteolytic activity correlated with the nitrite concentration in the San Juan River in 2016, which could be caused by the increase of ammonium concentration due to proteolytic activity. This ammonium is then available for oxidation reactions by another part of the microbial community in which nitrite is obtained. However, these results do not agree with those obtained by Traving et al. (2016). In the correlation analysis, a positive correlation was also found between proteolytic activity and the concentration of chlorophyll *a* in the San Juan River in 2017. That correlation indicates that with the increase in enzyme activity, the inorganic nutrients are used by cyanobacteria for their growth (Traving et al. 2017) and they also can produce proteolytic enzymes. This agrees with the results obtained by Admiraal and Tubbing (1991).

Proteolytic enzyme activity in freshwater ecosystems depends on physicochemical factors such as temperature, salinity, pH, the presence of humic material, β-UV radiation, the concentration of carbon dioxide, the concentration and dilution of nutrients, and biological variables such as the presence of biofilm-forming bacteria (Cunha et al. 2010; Giraldo et al. 2014). For this reason, it can be argued that the lack of correlation between the quality indicators

evaluated in this study and the proteolytic enzyme activity in the Almendares River does not indicate that there is no relationship between these variables, but that the proteolytic activity it is not directly affected by the indicators that were measured in this study. We can speculate that the proteolytic activity in the Almendares River could be more related to other pollutants that were not measured, such as heavy metals and humic matter, which can inhibit enzyme activity. On the other hand, in the San Juan River, there was a correlation between the proteolytic enzyme activity and some quality indicators evaluated, which indicates that in little impacted ecosystems the activity of proteases is directly related to nutrients of natural origin mainly from the decomposition of leaves, phytoplankton products, and runoff.

5 Conclusions

The presence of proteolytic microorganisms in the Almendares and San Juan rivers does not always correspond to the activity of proteolytic enzymes, which indicates that their activity depends on different biotic and abiotic factors. Most of the proteolytic bacterial in both rivers were Gram-positive bacilli endospore-forming, which shows the predominance of species belonging to the *Bacilli* class. Different physicochemical and microbiological factors are related to the proteolytic enzyme activity in the San Juan River according to the study period. On the other hand, this relationship has not been detected in the Almendares River, which indicates that the proteolytic activity could be affected by other pollutants discharged into its waters different from the ones evaluated in this study. The greatest proteolytic activities were detected in the San Juan River compared to the Almendares River, which indicates that in the most contaminated ecosystems, the proteolytic enzyme activity decreases compared to another less impacted system. The highest proteolytic activity was obtained in 2016 compared to 2017 in both rivers, which evidences their annual variation. During the last decades, human activities have had a strong negative impact on the quality of rivers, so it is urgent to understand their natural structure and functionality and thus be able to restore the ecological status of these ecosystems. For this reason, studies related to enzyme activity are essential to understand the complex dynamics related to nutrient metabolism.

Compliance with Ethical Standards

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