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ARTÍCULO ORIGINAL

Bacterial community structure of Almendares and San Juan rivers. Relationship with water quality

Estructura de la comunidad bacteriana de los ríos Almendares y San Juan. Relación con la calidad de sus aguas

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ABSTRACT

The combination of physicochemical and microbial indicators with bacterial community structure can be useful in the water quality evaluation. In the present study three sampling stations from Almendares river in Havana and three stations from San Juan river, Las Terrazas, Artemisa, were analyzed in order to evaluate the physicochemical and microbiological quality of both rivers, to analyze the bacterial community structure by denaturing gradient gel electrophoresis (DGGE) and to determine the relationship between the bacterial community structure and the physicochemical and microbiological water quality. The variables temperature, pH, inorganic nutrients concentrations, chemical oxygen demand, dissolved oxygen, total dissolved solids, precipitations, thermotolerant coliforms, *E. coli* and enterococci concentrations were determined. By DGGE of 16S rDNA, differences in the bacterial community structure of Almendares River stations with respect to San Juan River stations were detected. The temporal variability in the bacterial community structure was observed in both rivers. Fecal contamination was the main factor that influenced bacterial community structure in Almendares River. However, dissolved oxygen and precipitations were the main factors that influenced bacterial community structure in San Juan River.

Keywords: Water quality index, Denaturing gradient gel electrophoresis (DGGE), freshwater ecosystems, tropical environment, multivariate analysis

RESUMEN

La combinación de indicadores físico-químicos y microbianos con la estructura de la comunidad bacteriana puede ser útil en la evaluación de la calidad del agua. En el presente estudio, se analizaron tres estaciones de muestreo del río Almendares en La Habana y tres estaciones del río San Juan, Las Terrazas, Artemisa, con el objetivo de evaluar la calidad físico-química y microbiológica del agua de ambos ríos, analizar la estructura de la comunidad bacteriana mediante electroforesis en gel con gradiente desnaturalizante (DGGE) y determinar la relación entre la estructura de la comunidad bacteriana y la calidad

Recibido: 2020-01-25

Aceptado: 2020-10-17

físico-química y microbiológica del agua. Se determinaron las variables temperatura, pH, concentraciones de nutrientes inorgánicos, demanda química de oxígeno, oxígeno disuelto, sólidos totales disueltos, precipitaciones, concentraciones de coliformes termotolerantes, E. coli *y enterococos. Mediante la DGGE del 16S rDNA, se detectaron diferencias en la estructura de la comunidad bacteriana de las estaciones del río Almendares con respecto a las estaciones del río San Juan. La variabilidad temporal en la estructura de la comunidad bacteriana se observó en ambos ríos. La contaminación fecal fue el principal factor que influyó en la estructura de la comunidad bacteriana en el río Almendares. Sin embargo, el oxígeno disuelto y las precipitaciones fueron los principales factores que influyeron sobre la estructura de la comunidad bacteriana en el río San Juan.*

Palabras clave: *índice de calidad del agua, electroforesis en gel con gradiente desnaturalizante (DGGE), ecosistemas de agua dulce, ambientes tropicales, análisis multivariado.*

INTRODUCTION

Bacterial community can be useful for the water quality monitoring, because they experiment variations in their structure and composition according to environmental modifications or global climate change (Lear *et al.*, 2012; Washington *et al.*, 2013). The combination of environmental data with bacterial community information may offer an additional tool for assessing ecological status of freshwater ecosystems (Newton and McMahon, 2011; Newton and McLellan, 2015).

Culture-independent methods such as: Denaturing Gradient Gel Electrophoresis (DGGE), the Terminal Restriction Fragment Length Polymorphism (T-RFLP) and the Automated Ribosomal Intergenic Spacer Analysis (ARISA), have been used for the analysis of bacterial community structure. Particularly the DGGE allows obtaining taxonomic information, because the bands obtained can be split, reamplified and sequenced. Thus, this technique combines the advantages of cloning, sequencing and T-RFLP and can be used for the analysis of bacterial communities of different ecosystems (Duarte *et al.*, 2012; Liu *et al.*, 2012).

In the present investigation, two rivers from western Cuba have been evaluated, Almendares River (an urban river) and San Juan River (a rural river). Almendares River is located at Havana and is one of the most important streams at the Capital. This watercourse is highly polluted due to the constant dumping of wastewater from industrial and domestic origin (Arpajón *et al.*, 2011; Carballo *et al.*, 2011; Graham *et al.*, 2011; Knapp *et al.*, 2012; Romeu *et al.*, 2015b). On the other hand, San Juan River is located in Las Terrazas, Artemisa province, in the northeastern part of the Sierra del Rosario Biosphere Reserve.

This freshwater ecosystem presents good water quality (Arpajón *et al.*, 2012; Larrea *et al.*, 2013; Romeu *et al.*, 2015a) and its waters are considered mineral-medicinal (Peña *et al.*, 2001).

Bacterial community structure has been poorly evaluated in both rivers (Larrea *et al.*, 2014; Larrea, 2016). This type of investigation could be useful for bioremediation strategies in Almendares River, and for water conservation at Sierra del Rosario Biosphere Reserve. Taking into consideration these aspects, this study aims to evaluate the physicochemical and microbiological quality of Almendares and San Juan rivers, to analyze the bacterial community structure by denaturing gradient gel electrophoresis (DGGE) and to determine the relationship between the bacterial community structure and the physicochemical and microbiological quality.

MATERIALS Y METHODS

Sampling and environmental data

Punctual samples were collected from three sampling stations in Almendares River (Río Cristal [RC], Paila [P] and Puente de Hierro [PH]) on February, April, and June 2013. Other three sampling stations were sampled from San Juan River (Presa El Palmar [S-1], Presa San Juan [S-2] and Baños del San Juan [S-3]) on February, April and July 2013. These sampling stations were selected according to previous characterization of both rivers (Larrea *et al.*, 2013; 2014).

For sample collection, sterile plastic sampling bottles (1 L) were used (APHA, 2012). All samples were collected by triplicates. Water samples were taken about one meter from the shore and 15 cm deep.

Field parameters consisting in electrical conductivity, pH, temperature, total dissolved solid (TDS) and dissolved oxygen (DO) were measured at each sample location with the use of a multimeter (Mettler Toledo). All water samples were transported on ice to the laboratory and analyzed within 12 h.

Subsamples of water used for nutrients analysis (PO_4^{3-}-P, NH_4^+-N, NO_2^--N, and NO_3^--N) and chemical oxygen demand (COD) were filtered with oven-dried GF/F filters (Whatman International Ltd., Maidstone, United Kingdom) according to the standard French protocols AFNOR (2009).

Determination of thermotolerant coliforms, *Escherichia coli* and enterococci

Thermotolerant coliforms (ThC) were quantified by membrane filtration (0.45 µm-pore-size, 47-mm-diameter sterile cellulose nitrate filters, Sartorius) on Lactose agar with Tergitol (0.095 ‰ wt/vol final concentration) and triphenyl 2,3,5-tetrazolium chloride (TTC) (0.024 ‰ wt/vol final concentration) (AFNOR, 2009), *Escherichia coli* (EC) and enterococci (Ent) were quantified by the same method on Coliform Chromocult agar and Enterococci Chromocult agar respectively according to Ouattara *et al*. (2014). The bacteria were cultivated 24 h at 37 °C for EC and Ent and 24 h at 44 °C for ThC. Orange colonies producing a yellow halo under the membrane after incubation were considered thermotolerant coliforms colonies, dark-blue colonies were considered *E. coli* colonies and red colonies were considered enterococci. Counts were expressed as colony-forming units (CFU) per 100 mL of sample.

Analysis of bacterial communities of Almendares and San Juan rivers

DNA extraction and amplification of 16S rDNA fragments

Microbial biomass was collected from the water column and concentrated by filtration. Aliquots of each sample (0.5 L for Almendares River stations and 1 L for San Juan River stations) were filtered in triplicate through cellulose nitrate filters of 5 µm and consequently through cellulose nitrate filters of 0.22 µm. From 0.22 µm filters, DNA extraction was performed by SDS-phenol-chloroform method (Oh *et al.*, 2011). All DNA samples were purified by DNeasy Blood and Tissue Kit (Qiagen).

In order to increase the sensitivity and to facilitate DGGE analysis, nested PCR technique was performed. During the first amplification the PCR tube containing 25 µL of the reaction mixture consisting of a concentration of 1 X of OneTaq Quick-Load 2X Master Mix mixed with standard buffer (BioLabs, New England), a final concentration of 0.2 µM of the universal primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´ -TACCTTGTTACGACTT-3´) for amplifying 16S rDNA gene (Zhou *et al.*, 2009), 1 µL template DNA (~70 ng) and sterile MilliQ water. PCR conditions were as follows: pre-heating at 94 °C for 5 min; 30 cycles of denaturing at 94 °C for 30 s; annealing at 55 °C for 30 s and extension at 72 °C for 5 min, and a final extension at 72 °C for 7 min. From the products of the first amplification reaction, two independent amplification reactions were performed, one with primers 341FGC clamp (5´- GCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGGAGG-3´) and 534R (5´-ATT ACCGCGGCTGCTGG-3´) which amplify the V3 variable region of 16S rDNA of *Bacteria* domain (Muyzer *et al.*, 1993) and the other with the primers 517FGC (5´-GTGCCAGCAGCCGCGG-3´) (Gich *et al.*, 2005) and 1061R (5´-CACGACACGAGCTGACGAC-3´) (Degnan and Ochman, 2012) which amplify the V4-V6 variable regions of 16S rDNA of *Bacteria* domain. Both reactions were performed in a final volume of 50 µL of the reaction mixture containing a concentration of 1 X Quick-OneTaq of Load Master Mix with 2 X standard buffer (BioLabs, New England), 0.2 µM of the primers, 1 µL of the amplified product and sterile MilliQ water. Thermal cycling conditions were as follows: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 53 °C for 1 min and 2 min at 72 °C and a final extension step of 7 min at 72 °C. All PCR reactions were performed in a Thermal Cycler (ThermalCycler C 1000 TM BIO-RAD).

DGGE and sequencing of predominant bands

DGGE was performed using the system Ingeny phor U (Rafer, Spain). The PCR products obtained from the reaction with primers 341FGC and 534R were loaded onto a 10% polyacrylamide gel (w/v) in TAE 1X buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 8), while the products obtained from the reaction with primers 517FGC and 1061R were loaded onto a 7% polyacrylamide gel. Polyacrylamide gels were made with a denaturing gradient of 20-70% (where 100% denaturant containing 7 Molar urea and 40% formamide) (Duarte *et al.*, 2012).

All electrophoresis were performed at a voltage of 200 V for 6 h and 30 min at 60 °C. Gels were stained with SYBR Gold (1:10 000 dilution; FMC BioProducts, Rockland, ME, USA) for one hour. Stained gels were photographed on a UV transilluminator cabinet with a video camera (Alpha Innotech Multimage Light Cabinet Positions Filter).

To know the identity of the predominant bacterial populations, from the gels obtained with products of the reaction with 517FGC and 1061R primers, the most intense bands present at different sampling stations were cut. Selected bands were placed in Eppendorf tubes with sterile MilliQ water. The tubes were placed in refrigeration at 4 °C overnight and latter DNA fragments were amplified in a final volume of 25 µL of the reaction mixture consisting of a concentration of 1 X of OneTaq Quick-Load mixture 2X Master Mix with standard buffer (BioLabs, New England), a final concentration of 0.2 µM from the 517F (5´-CC AGCAGCCGCGGTAA-3´) (Gich *et al.*, 2005) and 1061R primers, 1 µL template DNA of the extracted band and sterile MilliQ water. After an initial denaturation step of 5 min at 94 °C, samples were amplified for 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C and one final extension step of 5 min at 72 °C.

The reaction products were purified and sequenced in Macrogen Europe (Amsterdam, Netherlands). The sequence analysis was performed by comparing obtained sequences with the reference sequences in the National Center for Biotechnology Information USA (NCBI), through the BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1990).

Statistical analyses

Based on the presence (1) or absence (0) of individual bands in each lane, a binary matrix was constructed using the program PyElph 1.4 (Pavel and Vasile, 2012), and a comparative analysis of DGGE profiles was carried out with PRIMER 6 software (PRIMER-E, Ltd., United Kingdom) (Clarke and Gorley, 2006). Cluster analysis using Sorensen similarity coefficient and average linkage technique by the method of unweighted pair group method with arithmetic mean (UPGMA) was performed.

To investigate the relationships between bacterioplankton community and environmental variables in Almendares and San Juan rivers, a direct-gradient approach was used, i.e. a redundancy analysis (RDA) using the software package CANOCO, version 4.5 for Windows (ter Braak and Šmilauer, 2002). This method was chosen after initial analysis by detrended correspondence analysis (DCA), which revealed that the data exhibited a linear response to the environmental variables. Data were imported using the WCanoImp program within the CANOCO package. Then was used the CANOCO program to perform RDA with species scaling on intersample distances so that samples and environmental variables formed a biplot. Explanatory variables were added until further addition of variables failed to contribute significantly ($p<0.05$) to a substantial improvement to the model's explanatory power. To statistically evaluate the significance of the first canonical axis and of all canonical axes together, was used a Monte Carlo permutation test with 999 unrestricted permutations under the reduced model.

Finally, to represent biplots, was used the program CANODRAW within the CANOCO package. Spearman's rank pairwise correlations between the different sets of tested environmental variables mentioned above were performed before RDA in order to remove covarying variables.

Calculation of Water Quality Index (WQI)

The Water Quality Index (WQI) was calculated according to Yisa *et al*. (2010). First the quality rating scale for each parameter qi was calculated by using this expression:

$qi = (Ci/Si)\times 100$

where (Ci) is the concentration of each parameter in each water sample and (Si) is the standard permissible value for each indicator giving by WHO (2003). Then the relative weight (Wi) was calculated by a value inversely proportional to the recommended standard (Si) of the corresponding parameter:

$Wi = 1/Si$

Finally, the overall WQI for drinking purposes is considered and permissible WQI for the drinking water is taken as 100:

$\text{Overall WQI} = \Sigma qiWi/\Sigma Wi$

WQI was calculated from the point of view of the suitability of surface water for human consumption (Atulegwu and Njoku, 2004). According to these authors there are five categories for water quality: excellent water quality (<50), good water quality (50-100), poor water quality (100-200), very poor water quality (200-300) and water unsuitable for drinking purposes (>300).

RESULTS

Physicochemical and microbiological quality of Almendares and San Juan rivers

Physicochemical and microbiological water quality of Almendares and San Juan rivers in the period February-July 2013 is listed in Table 1. Based on their electrical conductivity, phosphate and nitrate concentrations, Almendares River sampling stations can be considered as hypereutrophic, and San Juan River sampling stations can be considered oligo/ mesotrophic according to the classification of Sigee (2005). The concentrations of nitrate, nitrite, ammonium, phosphate, total dissolved solids, *E. coli*, thermotolerant coliforms and enterococci were always higher in Almendares River compared with San Juan River ($p<0.01$). Paila station in Almendares River was the most polluted according to the results of bacterial indicators of fecal contamination, ammonium and dissolved oxygen concentrations.

Table 1. Environmental data recorded for Almendares and San Juan rivers sampling stations.
Tabla 1. *Datos ambientales registrados para estaciones de muestreo de los ríos Almendares y San Juan.*

	Almendares River			**San Juan River**		
Environmental data[a]	**RC**	**P**	**PH**	**S-1**	**S-2**	**S-3**
Temperature (ºC)	20.7-27.9	21.0-27.0	24.4-30.2	23.7-26.9	23.3-26.1	22.3-25.9
pH	6.8-7.4	7.1-7.6	7.1-7.6	6.6-7.7	6.5-7.8	6.7-7.9
Electrical conductivity (µS.cm^{-1})	670-799	1090-1338	3730-5310	280-4190	360-3800	500-4360
TDS (mg.L^{-1})	380-450	669-760	1500-3500	190-2060	240-1907	330-2210
DO (mg.L^{-1})	0.9-2.8	0.6-2.2	3.7-4.8	6.0-7.7	5.4-7.1	5.2-6.8
COD (mg.L^{-1})	50.0-145.0	56.7-290.0	206.7-6368	15.0-105.0	58.3-83.3	5.0-195.0
NO_3-N (mg.L^{-1})	1.8-9.8	2.4-9.1	16.0-71.8	0.9-1.6	0.7-1.9	3.2-9.5
NO_2-N (mg.L^{-1})	0.02-0.08	0.37-0.40	1.0-1.9	0.0-0.002	0.0-0.01	0.02-0.06
NH_4-N (mg.L^{-1})	0.2-0.4	4.5-5.9	2.1-3.2	0.06-0.2	0.05-0.1	0.06-0.09
PO_4-P (mg.L^{-1})	0.1-0.3	2.3-4.6	0.8-2.1	0.02-0.03	0.0-0.01	0.0-0.1
Log (ThC.100 mL^{-1})	3.0-3.5	6.7-6.9	5.1-6.0	1.0-3.2	1.1-3.4	2.4-3.0
Log (EC.100 mL^{-1})	0.0-3.0	6.3- 6.7	5.0-5.3	0.0-3.0	0.0-2.5	2.0- 2.7
Log (Ent.100 mL^{-1})	2.0-3.0	5.9-6.0	3.4-4.0	0.0-2.5	0.0-2.8	2.3-2.6
Precipitations[b] (mm)	0.0-36.0	0.0-36.0	0.0-36.0	24.4-242.1	24.4-242.1	24.4-242.1

[a] In the table are represented the minimal and maximum values of each indicator determined in Almendares River (February-June 2013) and San Juan River (February-July 2013).

[b] In the case of Almendares River the data were obtained from the meteorological station of Casablanca, Institute of Meteorology, Havana. For San Juan River, the data were obtained from the Ecological Station Sierra del Rosario, Artemisa. It was taking into account the precipitations of 10 days before the sampling date.

TDS: Total Dissolved Solids; DO: Dissolved oxygen; COD: Chemical oxygen demand; ThC: Thermotolerant coliforms; EC: *Escherichia coli;* Ent: Enterococci.

RC: Río Cristal station; P: Paila station; PH: Puente de Hierro station; S-1: Presa El Palmar station; S-2: Presa San Juan station; S-3: Baños del San Juan station.

In San Juan River, all sampling stations had good microbiological water quality according to the Cuban guidelines (Norma Cubana 22, 1999). However, in Baños del San Juan station throughout the sampling period, and Presa El Palmar and Presa San Juan stations in July, it was found that concentrations of fecal contamination indicators exceeded the limits established by the Cuban Standard 22 for direct contact with the water. Significant differences among the mean concentrations of bacterial indicators of fecal contamination in the analyzed stations were not observed.

According to the WQI, the water from Río Cristal station in Almendares River is classified as poor water quality (see Materials and Methods 2.5). However, the waters from stations Paila and Puente de Hierro are unsuitable for drinking purposes (Table 2).

In San Juan River, the water of the stations S-1 and S -2 can be classified as excellent water quality (Table 3). Nevertheless, the waters from station S-3 are classified as good water quality.

Table 2. Computed WQI values for Almendares river (February-June 2013).

Tabla 2. *Valores computados de WQI para el río Almendares (febrero-junio de 2013).*

Physicochemical data	Standard permissible value (Si)	Overall WQI		
		RC	P	PH
pH	6.5-8.5	147.1	2579.2	1569.5
Conductivity (µs.cm^{-1})	20.0-1500.0			
TDS (mg.L^{-1})	500.0			
DO (mg.L^{-1})	4.0			
COD (mg.L^{-1})	10.0			
NO_{3}-N (mg.L^{-1})	10.0			
NO_2-N (mg.L^{-1})	0.5			
NH_4-N (mg.L^{-1})	0.5			
PO_4-P (mg.L^{-1})	0.1			

TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Oxygen; RC: Río Cristal station; P: Paila station; PH: Puente de Hierro station.

Table 3. Computed WQI values for San Juan river (February-July 2013).

Tabla 3. *Valores computados de WQI para el río San Juan (febrero-junio de 2013).*

Physicochemical data	Standard permissible value (Si)	Overall WQI		
		S-1	S-2	S-3
pH	6.5-8.5	32.3	17.4	50.1
Conductivity (µs.cm^{-1})	20.0-1500.0			
TDS (mg.L^{-1})	500.0			
DO (mg.L^{-1})	4.0			
COD (mg.L^{-1})	10.0			
NO_{3}-N (mg.L^{-1})	10.0			
NO_2-N (mg.L^{-1})	0.5			
NH_4-N (mg.L^{-1})	0.5			
PO_4-P (mg.L^{-1})	0.1			

TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Oxygen; S-1: Presa El Palmar station; S-2: Presa San Juan station; S-3: Baños del San Juan station.

Analysis of bacterial communities from Almendares and San Juan rivers by DGGE

By analyzing the bacterial community structure in the period February-July 2013 (Fig.1a) could be observed from the cluster analysis, the formation of four groups with a similarity of 60% (Fig.1b).

The first group includes the samples from RC station collected in April and June 2013, the second group its formed by the samples from P and PH stations collected in June 2013, the third group consists of samples from S-1 and S-3 stations collected in July 2013 and the fourth group includes the samples from S-1 and S-3 collected in the months of April 2013 and February 2013 respectively. The remaining samples were separated. PERMANOVA analysis indicated the existence of significant differences between Almendares and San Juan rivers ($p<0.01$), among sampling stations in each river ($p<0.05$) and among the sampling months ($p<0.05$).

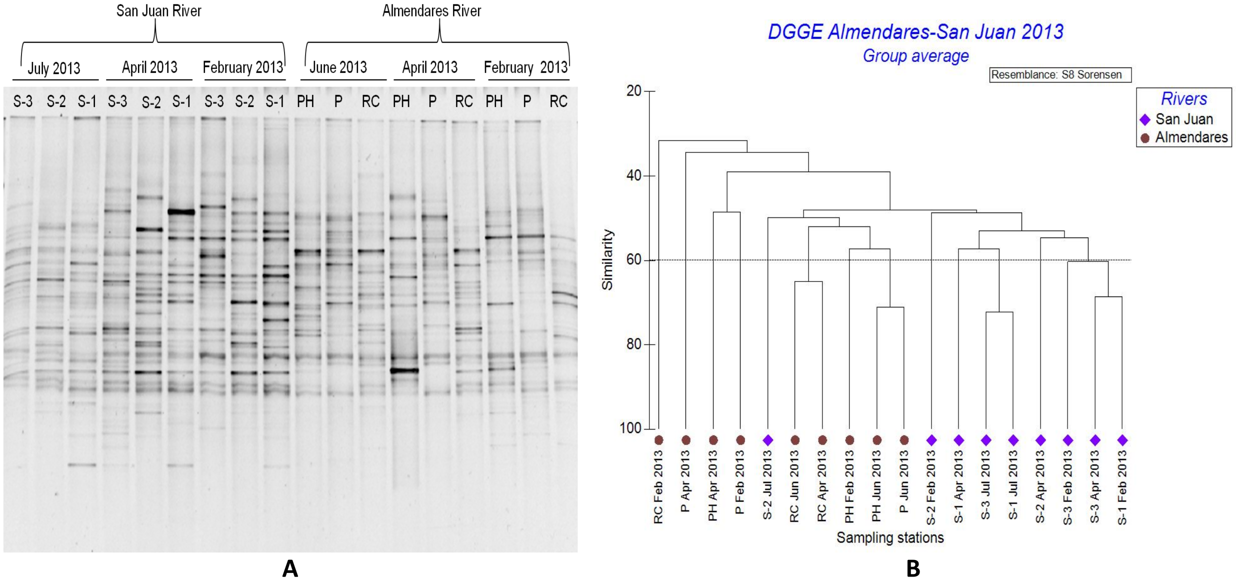

Figure 1. DGGE analysis of 16S rDNA fragments amplified by PCR with primers 341FGC and 534R. (A) Image of the gel obtained from samples of Almendares and San Juan rivers collected in the period February 2013-July 2013. Denaturant gradient used: 20-70% (denaturalizant 100 % is formed by urea 7 M and formamide 40 %). (B) Cluster analysis by UPGMA method based on band patterns of samples from Almendares and San Juan rivers. The similarity was expressed as percentage of Sorensen index. San Juan River sampling stations: S-3: Baños del San Juan; S-2: Presa San Juan; S-1: Presa El Palmar. Almendares River sampling stations: RC: Río Cristal; P: Paila; PH; Puente de Hierro.

Figura 1. *Análisis de los fragmentos de rADN 16S amplificados por PCR con los cebadores 341FGC y 534R por DGGE. (A) Imagen del gel obtenido de muestras de los ríos Almendares y San Juan en el período febrero 2013-julio 2013. Gradiente desnaturalizante utilizado: 20-70% (el desnaturalizante 100% está formado por urea 7 M y formamida 40%). (B) Análisis de conglomerados por el método UPGMA basado en los patrones de bandas de muestras de los ríos Almendares y San Juan. La similitud se expresó como porcentaje del índice de Sorensen. Estaciones de muestreo del río San Juan: S-3: Baños del San Juan; S-2: Presa San Juan; S-1: Presa El Palmar. Estaciones de muestreo del río Almendares: RC: Río Cristal; P: Paila; PH; Puente de Hierro.*

From the DGGE obtained by amplifying the 16S rDNA samples of Almendares River, using the 517FGC and 1061R primers, 10 bands were cut (Fig. 2, Table 4). Five bands were identified as *Gammaproteobacteria* class, two bands as *Betaproteobacteria* class, a band as *Flavobacterium* class, a band was related to the phylum *Actinobacteria* and a band was related to the phylum *Chloroflexi.* In the case of San Juan River, 12 bands were selected (Fig. 3, Table 5). Six bands related to *Actinobacteria* class, five bands related to *Betaproteobacteria* class and a band related to *Bacilli* class, were identified.

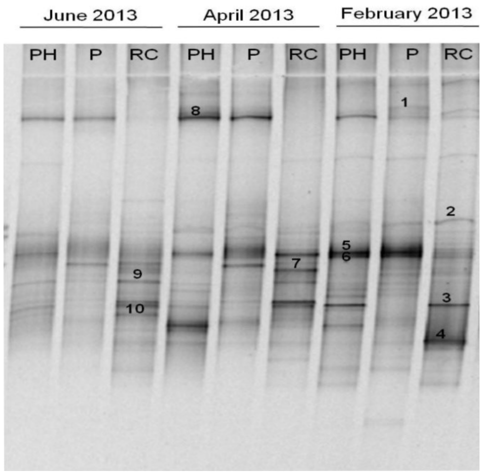

Figure 2. DGGE of 16S rDNA fragments amplified by PCR with primers 1061R 517FGC from samples collected in Almendares River in the period February-June 2013. Denaturant gradient used: 20-70% % (denaturalizant 100 % is formed by urea 7 M and formamide 40 %). Sampling stations: PH: Puente de Hierro; P: Paila; RC: Río Cristal. Numbers indicate selected bands for subsequent sequencing and identification.

Figura 2*. DGGE de fragmentos de rADN 16S amplificados por PCR con los cebadores 1061R 517FGC de muestras del río Almendares en el período de febrero a junio de 2013. Gradiente desnaturalizante utilizado: 20-70%% (desnaturalizante 100% formado por urea 7 M y formamida 40%). Estaciones de muestreo: PH: Puente de Hierro; P: Paila; RC: Río Cristal. Los números indican las bandas seleccionadas para su posterior secuenciación e identificación.*

Table 4. Identification of selected bands from DGGE of 16S rDNA fragments amplified by PCR with primers 1061R 517FGC from samples collected in Almendares river in the period February-June 2013.

Table 4. *Identificación de bandas seleccionadas de DGGE de fragmentos de rADN 16S amplificados por PCR con cebadores 1061R 517FGC de muestras del río Almendares en el período de febrero a junio de 2013.*

Bands	Identification	Phylum	Class	Percentage of similarity (%)
1	*Serratia* sp.	*Proteobacteria*	*Gammaproteobacteria*	99
2	*Serratia liquefaciens*	*Proteobacteria*	*Gammaproteobacteria*	99
3	*Cloroflexi* non culturable	*Cloroflexi*	-	90
4	*Gammaproteobacteria*	*Proteobacteria*	*Gammaproteobacteria*	97
5	*Acinetobacter oleivorans*	*Proteobacteria*	*Gammaproteobacteria*	99
6	*Acinetobacter* sp.	*Proteobacteria*	*Gammaproteobacteria*	98
7	*Actinobacteria* non culturable	*Actinobacteria*	-	84
8	*Flavobacterium* sp.	*Bacteroidetes*	*Flavobacteria*	83
9	*Variovorax* sp.	*Proteobacteria*	*Betaproteobacteria*	90
10	*Variovorax* sp.	*Proteobacteria*	*Betaproteobacteria*	90

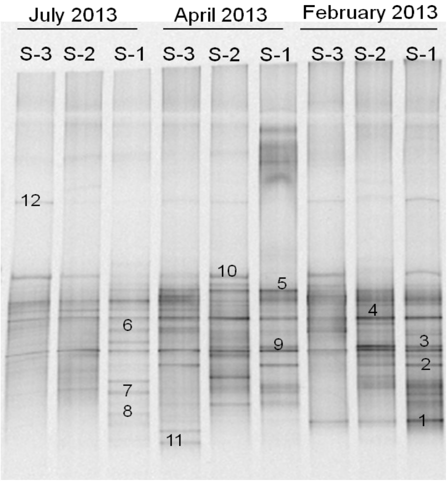

Figure 3. DGGE of 16S rDNA fragments amplified by PCR with primers 1061R 517FGC from samples collected in San Juan River in the period February-July 2013. Denaturant gradient used: 20-70%. Sampling stations: S-3: Baños del San Juan; S-2: Presa San Juan; S-1: Presa El Palmar. The numbers indicate the selected bands for later sequence and identification.

Figura 3. *DGGE de los fragmentos de ADNr 16S amplificados por PCR con cebadores 1061R 517FGC de muestras recolectadas en el río San Juan en el período de febrero a julio de 2013. Gradiente desnaturalizante utilizado: 20-70%. Estaciones de muestreo: S-3: Baños del San Juan; S-2: Presa San Juan; S-1: Presa El Palmar. Los números indican las bandas seleccionadas para una secuencia e identificación posterior*

Table 5. Identification of selected bands from DGGE of 16S rDNA fragments amplified by PCR with primers 1061R 517FGC from samples of San Juan River collected in the period February-July 2013.

Tabla 5. *Identificación de bandas seleccionadas de DGGE de fragmentos de rDNA 16S amplificados por PCR con cebadores 1061R 517FGC de muestras del río San Juan en el período de febrero a julio de 2013.*

Bands	Identification	Phylum	Class	Percentage of similarity (%)
1	*Actinobacteria*	*Actinobacteria*	*Actinobacteria*	97
2	*Betaproteobacteria* non culturable	*Proteobacteria*	*Betaproteobacteria*	94
3	*Actinobacteria*	*Actinobacteria*	*Actinobacteria*	97
4	*Betaproteobacteria* non culturable	*Proteobacteria*	*Betaproteobacteria*	95
5	*Hydrogenophaga* sp.	*Proteobacteria*	*Betaproteobacteria*	97
6	*Bacillus* sp.	*Firmicutes*	*Bacilli*	98
7	*Actinobacteria* non culturable	*Actinobacteria*	*Actinobacteria*	98
8	*Arthrobacter* sp.	*Actinobacteria*	*Actinobacteria*	96
9	*Actinobacteria* non culturable	*Actinobacteria*	*Actinobacteria*	98
10	*Betaproteobacteria*	*Proteobacteria*	*Betaproteobacteria*	94
11	*Actinobacteria* non culturable	*Actinobacteria*	*Actinobacteria*	97
12	*Betaproteobacteria*	*Proteobacteria*	*Betaproteobacteria*	84

Analysis of the relationship between the bacterial community composition and the physicochemical and fecal contamination indicators

Precipitations (*p*=0.010) and dissolved oxygen (*p*=0.010) were the main factors that influenced the variability of the bacterial community structure in San Juan River. However, ammonium concentration (*p*=0.030) was the factor that most influenced bacterial community structure in Almendares River .

Monte Carlo test for all canonical axes (*p*=0.020) was significant, indicating that the selected variables can explain the variability of the bacterial community structure in both rivers. Precipitations, dissolved oxygen, ammonium concentration, total dissolved solids and chemical oxygen demand, explained 9.5 %, 9.0 %, 8.6 %, 6.1% and 5.5 % respectively, of the bacterioplankton structure variability in Almendares and San Juan rivers (Fig. 4, Table 6).

According to the redundancy analysis, the cumulative percentage of variance explained by the species-environmental variables indicated that the first and second canonical axes describe 60.4 % of the variance. Correlations species-environment from each axis, were 0.964 and 0.867 for the first and second axis respectively. Dissolved oxygen, TDS and ammonium concentration were the main components of the first axis.

Precipitations and COD were the main components of the second axis. Based on the pattern in the biplot, we divided the samples into seven clusters: Cluster 1: samples from all the stations of San Juan River collected in February 2013 and samples from S-1 station in April 2013, characterized by the highest values of dissolved oxygen and low values of COD and ammonium concentration. Cluster 2: samples from S-2 and S-3 stations (April 2013) characterized by medium concentrations of dissolved oxygen. Cluster 3: samples from San Juan River (July 2013) characterized by the highest values of precipitations. Cluster 4, included the samples from RC station (June 2013), P station (February 2013) and PH station (February 2013), characterized by the highest values of TDS and precipitations in Almendares River. Cluster 5: samples from P and PH stations (April and June 2013) characterized by the highest values of ammonium and COD concentrations. Cluster 6: samples from RC station (February 2013) with medium values of TDS and the lowest values of COD and ammonium concentration in Almendares River and Cluster 7: samples from RC station (April 2013) characterized by medium values of COD and low ammonium concentration. The samples from Almendares River were separated from the samples of San Juan River.

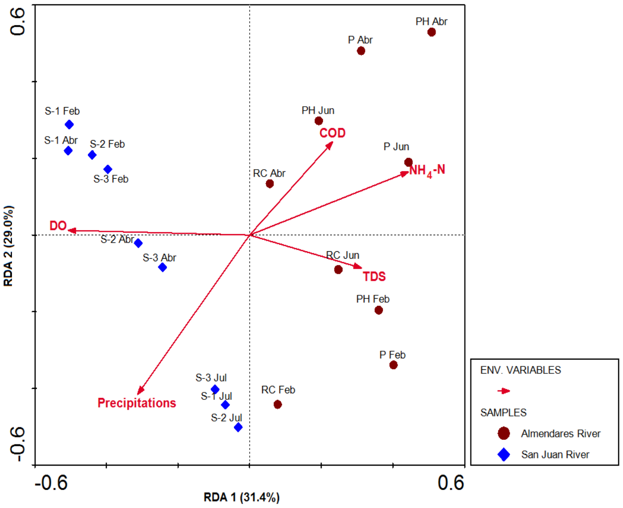

Figure 4. Biplot of redundancy analysis (RDA) of the bacterial community composition from Almendares and San Juan rivers in the period February-July 2013 using physicochemical and microbiological indicators. The red arrows indicate the direction in which the value of each indicator of water quality increases. The length of the arrows indicates the degree of correlation with the axis shown. The position of the sampling stations on the arrows is interpreted by the projection of the symbols on the arrows, and indicates the degree of influence of the water quality indicator represented by the red arrow on the bacterial community composition of the sampling station. DO: Dissolved Oxygen; TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; NH_4-N: ammonium concentration; RC: Río Cristal station; P: Paila station; PH: Puente de Hierro station; S-1: Presa El Palmar station; S-2: Presa San Juan station; S-3: Baños del San Juan station; Feb: February; Abr: April; Jun: June; Jul: July.

Figura 4. *Biplot de análisis de redundancia (RDA) de la composición de la comunidad bacteriana de los ríos Almendares y San Juan en el período de febrero a julio de 2013 utilizando indicadores fisicoquímicos y microbiológicos. Las flechas rojas indican la dirección en que aumenta el valor de cada indicador de calidad del agua. La longitud de las flechas indica el grado de correlación con el eje que se muestra. La posición de las estaciones de muestreo en las flechas se interpreta mediante la proyección de los símbolos en las flechas, e indica el grado de influencia del indicador de calidad del agua representado por la flecha roja en la composición de la comunidad bacteriana de la estación de muestreo. DO: Oxígeno disuelto; TDS: Sólidos disueltos totales; DQO: demanda química de oxígeno;* NH_4*-N: concentración de amonio; RC: estación Río Cristal; P: estación Paila; PH: estación Puente de Hierro; S-1: estación Presa El Palmar; S-2: estación Presa San Juan; S-3: estación Baños del San Juan; Feb: febrero; Abr: abril; Junio: junio; Jul: julio*

Table 6. Summary from redundancy analyses (RDA) results of the bacterioplankton community structure data when constrained by water quality indicators from Almendares and San Juan rivers.

Tabla 6. *Resumen de los resultados del análisis de redundancia (RDA) de los datos de la estructura de la comunidad del bacterioplancton influenciados por los indicadores de calidad del agua de los ríos Almendares y San Juan.*

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Total Variance**
Eigenvalues	0.110	0.102	0.076	0.041	1.000
Species-environment correlations	0.964	0.867	0.930	0.833	
Cumulative percentage variance:					
of species data	11.0	21.2	28.8	32.8	
of species-environment relation	31.4	60.4	82.0	93.6	
Sum of all eigenvalues					1.000
Sum of all canonical eigenvalues					0.351
Correlations with the axes					
Dissolved oxygen (DO)	**-0.8407**	0.0203	0.0479	0.5358	
Total dissolved solids (TDS)	0.5173	-0.1486	-0.0024	**0.8424**	
Ammonium concentration (NH_4-N)	**0.7351**	0.2839	0.4248	-0.1303	
Chemical Oxygen Demand (COD)	0.3843	0.4195	0.1524	0.4730	
Precipitations	-0.5194	**-0.7182**	0.3319	0.3178	

DISCUSSION

Physicochemical and microbiological quality of Almendares and San Juan rivers in the period February -July 2013

Oxygen is important for the survival of aquatic life. It is argued that in a good water quality river, the amount of dissolved oxygen should exceed four parts per million; however, when the analysis result is below this value indicates water pollution (De La Cruz, 2008), as occurs in Almendares River (Table 1). Low concentrations of dissolved oxygen in Almendares River are the result of suspended solids containing organic and inorganic materials which are oxidized at the expense of oxygen.

Total dissolved solids (TDS) and electrical conductivity both constitute an indirect measure of the salinity of a solution (Niekerk *et al.*, 2014). The highest values of both indicators during the study period were found in P and PH stations and in the stations of San Juan River in July 2013. This may be because PH station is located in the estuarine area of Almendares River where a mixture of freshwater from the river with seawater occurs, which contributes to increase salinity. However, P station is upstream; thus, high TDS and conductivity values may be due to the contamination at this sampling point. In San Juan River, high TDS and conductivity values in the month of July 2013 coincided with abundant rainfall, where large amount of sediments were washed down from the mountains to the waters. These sediments were detected during the sampling and constitute a supply of inorganic nutrients, minerals and organic compounds that tend to vary the conductivity of the water.

The highest values of inorganic nutrients were obtained in Almendares River, which exceeded the limits set by the World Health Organization (W.H.O, 2003). The high concentrations of dissolved inorganic nitrogen and phosphate in Almendares River, demonstrates the poor quality of this ecosystem characterized by murky waters and low dissolved oxygen concentrations. However, in San Juan River, waters are clear and it can be seen submerged macrophytes in S-1 and S-2 stations, typical of oligo/mesotrophic freshwaters environments as referred Guo *et al*. (2014).

Analyzing the microbiological quality of both rivers, it was observed that stations of Almendares River have the highest values of fecal contamination indicators compared to San Juan River.

Almendares River receives constants dumping of sewage waters that contribute to the pollution of this freshwater ecosystem (Romeu *et al.*, 2015b). However, San Juan River is located in a rural area that it is less influenced by antropogenic activities (Larrea *et al.*, 2013).

Considering the WQI in the period evaluated, the sampling stations from Almendares River had a poor water quality and are unsuitable for drinking purposes, results according to those of Romeu *et al*. (2015b). Nevertheless, the stations of San Juan River presented excellent or good water quality, which is in correspondence with studies previously done by Larrea *et al*. (2013).

In order to simplify, notify and understand the complex information obtained from a water body, different authors have calculated water quality indexes (Yisa and Jimoh, 2010; Rubio-Arias *et al.*, 2012; Rubio -Arias *et al.*, 2016). Thus, with a single number it can be interpreted the pollution level in the water bodies including several chemical and microbiological variables. It is important for the water quality analyses because the water quality index allows summarizing the information of most of these quality variables to simple expression, so that they can be easily transmitted to the entities responsible for the care, preservation and sanitation of aquatic ecosystems and they can take actions for their conservation (Samboni *et al.*, 2007; Yisa y Jimoh, 2010).

Relationship between the bacterial community structure and the physicochemical and fecal contamination indicators in Almendares and San Juan rivers

Based on the structure of bacterial communities, in the Almendares River it was observed that the months of April and June 2013 are separated from the month of February 2013 (Figures 1b and 4). The same happens in San Juan River where the month of July 2013 is separated from the months of February and April 2013. In addition it is observed how the stations of Almendares River are separated from the stations of San Juan River, which supports the idea that bacterial communities can be used as indicators of the ecological status of aquatic ecosystems (Lear *et al.*, 2012; Washington *et al.*, 2013). It is argued that bacterial communities are highly sensitive to environmental changes as well as to persistent changes in ecosystems, due to the pollution from human activity or global climate change (Lear *et al.*, 2008).

The increasing projections of the influence of the variables analyzed in different sampling stations coincide in several cases with differentiated water quality. It is interesting the fact that samples that belong to the same subgroup derived from the cluster analysis are distant if the influence of the chemical-physical parameters is analyzed. This behavior is due to their similarity in the composition and structure of the microbial communities. So, the high dissolved oxygen and low fecal indicators and ammonia are the best conditions for the bacterial community diversity. On the other hand, the temporal and spatial changes detected in the two freshwater ecosystems may be related to abiotic and biotic factors that regulate the changes of communities in aquatic ecosystems (Lindström *et al.*, 2005; Berdjeb *et al.*, 2011a; Berdjeb *et al.*, 2011b; Llirós *et al.*, 2014).

Through DGGE was most frequently identified *Gammaproteobacteria* class in Almendares River. The *Gammaproteobacteria* class is found most frequently in saltwater environments (eg, brackish oceans and lakes) and is less abundant in freshwater environments where they are considered pollutants. It is considered that the bacteria of this class are transitory members of the freshwater ecosystems brought from other environments, as is the case of enteric bacteria (Newton *et al.*, 2011). This fact denotes the influence of fecal contamination on the bacterial communities' structure of the Almendares River. However, the *Actinobacteria* class, which represents one of the dominant groups in aquatic ecosystems and constitutes more than 70% of the bacterioplankton of globally distributed rivers and lakes (Tang *et al.*, 2009; Newton *et al.*, 2011; Ghai *et al.*, 2014), was most frequently detected in San Juan River. Previously it has been detected that the class *Actinobacteria* is in greater proportion compared to the class *Gammaproteobacteria* in freshwater ecosystems with a low level of contamination (García-Armisen *et al.*, 2014; Llirós *et al.*, 2014) as is the case of the San Juan River. The rest of the class detected in both rivers can be found in different freshwaters ecosystems (Newton *et al.*, 2011; Kaushik *et al.*, 2014).

The factor that most influenced the structure of bacterial community in Almendares River was ammonium concentrations, an indicator of fecal contamination. Similar results were obtained in this river in the period October 2011-April 2012 (Larrea *et al.*, 2014), denoting that the conditions of this freshwater ecosystem have not change. The constant dumping of wastewater to the river contributes to the predomination of anaerobes or facultative anaerobes microorganisms as *Gammaproteobacteria* class members. At the same time, the low dissolved oxygen concentration enhances this situation. Instead, in San Juan River, high concentrations of dissolved oxygen and precipitations are the main factors that influenced bacterial community structure. This freshwater ecosystem is located in a rural area in Sierra del Rosario, a protected area; where a sustainable development program is carry out. Besides, San Juan River is less impacted by anthropogenic activities compared with Almendares River, because there are not industries in this region.

Dissolved oxygen is an indicator of good water quality that favors the development of aerobic organisms (De La Cruz, 2008) as *Actinobacteria* class members, detected in the present study. On the other hand, precipitations can contribute to the drainage and the trawling of the soils (Crim *et al.*, 2012), as happened during the month of July 2013, where 242.1 mm of precipitation were reported. This soil input can contribute not only to the entry of bacteria into the river waters, but also to the entry of organic compounds, all of which promotes changes in the structure of bacterial communities (Becker *et al.*, 2016). In the present study, was appreciated how natural and anthropogenic factors can impact bacterial community structure in rural and urban freshwater ecosystems. This information is useful for the management and preservation of freshwaters ecosystems and for future bioremediation actions that could be carried out in Almendares River.

Finally, at San Juan River stations, high concentrations of dissolved oxygen, precipitations and low concentrations of fecal contamination were related with bacterial community structure. However in Almendares River, high concentrations of fecal contamination and chemical oxygen demand were the main factors that influenced bacterial community structure. These results contribute to the diagnosis of the aquatic ecosystems analyzed.

Using DGGE, it was possible to understand relationships between bacterial community and environmental variables in a rural and an urban river. It is demonstrated that the composition of the bacterial communities in the freshwater ecosystems constitutes a response to the environmental changes.

The analysis herein has provided useful information on the role of certain environmental variables that shape community structure in rivers.

ACKNOWLEDGMENTS

This work was supported by the international cooperation project from Walloon region and the PNCB-CITMA Project. The authors want to thanks Dr. Maickel Armenteros and Dr. José L. Ponce de León for their assistance with the statistics. Also, the authors want to thanks Fidel Hernández and Jorge Luis Zamora, for the help with the access to the Biosphere Reserve Sierra del Rosario, Artemisa, Cuba.

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