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ARTÍCULO ORIGINAL

Structure of rapid growth culturable heterotrophic bacterial community in Almendares River, Havana, Cuba

Estructura de la comunidad bacteriana heterotrófica cultivable de rápido crecimiento en el río Almendares, La Habana, Cuba

Jeny A. Larrea Murrell*, Marcia M. Rojas Badía, Daysi Lugo Moya, Mayra Heydrich Pérez

Departamento Microbiología, Facultad de Biología, Universidad de La Habana, Cuba.

*Autor para correspondencia: *adina@fbio.uh.cu*

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ABSTRACT

Water pollution is a consequence of the industrialization and the society's development due to the constant pouring of domestic and industrial wastewater without a previous treatment; these facts contribute to the environment deterioration and the diversity lost. Three sampling stations of Almendares River (Havana, Cuba) with different pollution levels were studied for one year, to characterize the structure of rapid growth culturable heterotrophic bacterial community. For isolation, serial dilutions of water samples were grown in nutrient agar and incubated at 30°C for 72h. Representative different morphology colonies were isolated and purified on the same culture medium. A total of 60 isolates were obtained and were identified by API system as belonging to the genera *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Alcaligenes*, *Citrobacter* and *Pseudomonas*, prevailing the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria*. The genus *Arthrobacter* was isolated more frequently in the sampling station Paila, compared with the rest of the Almendares River stations, while the genera *Acinetobacter* and *Bacillus* were isolated more frequently in Río Cristal and Puente de Hierro stations. To characterize the bacterial community structure were used indexes of species richness (S*), Simpson (L') and Shannon-Wiener (H'). The indexes of Simpson and Shannon-Wiener showed significant differences among the bacterial diversity of the three sampling stations. No significant differences were detected by analyzing the species richness. The results showed the influence of pollution on bacterial diversity, which is significantly affected from upstream to downstream in Almendares River.

Keywords: diversity indexes, pollution, water quality, freshwater, *Bacilli*, bacterial diversity

RESUMEN

La contaminación del agua es una consecuencia de la industrialización y el desarrollo de la sociedad debido al vertido constante de aguas residuales domésticas e industriales sin un tratamiento previo; estos hechos contribuyen al deterioro del medio ambiente y la pérdida de la diversidad. Se estudiaron tres estaciones de muestreo del río Almendares (La Habana, Cuba) con diferente nivel de contaminación durante un año, con el objetivo de caracterizar la estructura de la comunidad bacteriana heterotrófica cultivable de rápido crecimiento. Para el aislamiento, se cultivaron diluciones seriadas de muestras de agua en Agar Nutriente y se incubaron a 30°C durante 72 h. Se

aislaron y purificaron las colonias representativas de diferente morfología en el mismo medio de cultivo. Se obtuvieron un total de 60 aislados que fueron identificados por el sistema API como pertenecientes a los géneros Acinetobacter, Arthrobacter, Bacillus, Micrococcus, Staphylococcus, Alcaligenes, Citrobacter *y* Pseudomonas*, predominando los filos* Firmicutes, Actinobacteria *y* Proteobacteria. Arthrobacter *se aisló con mayor frecuencia en la estación Paila, en comparación con el resto de las estaciones del río Almendares, mientras los géneros* Acinetobacter *y* Bacillus *se aislaron con mayor frecuencia en las estaciones Río Cristal y Puente de Hierro. Para caracterizar la estructura de la comunidad bacteriana se utilizaron los índices de riqueza de especies (S*), Simpson (L') y Shannon-Wiener (H'). Los índices de Simpson y Shannon-Wiener mostraron diferencias significativas entre la diversidad bacteriana de las tres estaciones de muestreo. No se detectaron diferencias en la riqueza de especies. Los resultados mostraron la influencia de la contaminación en la diversidad bacteriana, que se ve afectada significativamente de las estaciones corrientes arriba a las estaciones corrientes abajo en el río Almendares.*

Palabras clave: *índices de diversidad, contaminación, calidad de agua, agua dulce, Bacilli, diversidad bacteriana*

INTRODUCTION

Water pollution is a consequence of the industrialization and the society's development due to the constant pouring of domestic and industrial wastewater without a previous treatment; these facts contribute to the environment deterioration and the biodiversity lost (García-Armisen *et al*., 2014, Jordaan and Bezuidenhout, 2015; Kirschke *et al*., 2020). The microbial community plays an important role in the degradation of pollutants from human activity and contributes to the natural self-purification of water body (Wang *et al.*, 2016), but the anthropogenic contamination can negatively affect their structure and function (Connor *et al.*, 2014; Jordaan and Bezuidenhout; 2015, Ibekwe *et al.*, 2016; Wang *et al.*, 2016; Wang *et al*., 2019) .

Most bacteria found in aquatic environments proceed from soil sources and are carried to the water due to drag processes and soil drainage (Newton *et al.*, 2011). However, any water body has a bacterial community, although these may vary greatly in groups and number of cells (de Sousa and Silva-Sousa, 2001). The most frequently bacteria found in aquatic ecosystems, are species from the genera *Bacillus*, *Pseudomonas, Aeromonas, Citrobacter, Corynebacterium, Enterobacter, Escherichia, Flavobacterium, Klebsiella, Micrococcus, Proteus, Vibrio* and *Alcaligenes* (Azmuda *et al.*, 2019).

Globally, there is an increasing interest to monitor and restore freshwater ecosystems, in order to improve their ecological, recreational, cultural, educational and economic value (Lear *et al.*, 2009; Kirschke *et al.*, 2020). In this sense, bacterial communities are indicators that respond quickly to environmental changes as a result of its rapid life cycle (Lear *et al.*, 2008, Jordaan and Bezuidenhout, 2015, Linz *et al.*, 2017) and can be used to monitor spatial and temporal variability that occurs naturally in freshwater systems, as well as persistent changes in ecosystems due to pollution from human activity and global climate change (Lear *et al.*, 2008; Ibekwe *et al.*, 2016).

In aquatic ecosystems is important to evaluate changes in microbial community structure, because it constitutes the basis of the biogeochemical cycles of elements like carbon, nitrogen, and also play an important role in the biosphere (Newton *et al.*, 2011; Wang *et al.*, 2017; Azmuda *et al.*, 2019). To determine the community structure and diversity assessment, several mathematical indexes that describe the species richness within the community have been used. These indexes include species richness (S*), Simpson (L') and Shannon -Wiener (H') (Wang *et al.*, 2012; Xia *et al.*, 2014).

Microbial ecology in tropical areas has been studied using culturable dependent methods (Kenzaka *et al.*, 2001) as well as culturable independent methods (Gouvêa *et al.*, 2010; Larrea *et al.*, 2014; Inceoglu *et al.*, 2015; Mukherjee *et al.*, 2016; Azmuda *et al.*, 2019), but current knowledge is still limited. Culturable dependent methods have a limited capacity to characterize water microbiota compared with culturable independent methods; however, it allowed bacterial identifications to the species level, in contrast with the other procedures that rarely produced identifications below the order (Vaz-Moreira *et al.*, 2011). Historically, the diversity and physiology of microorganisms, has been assessed by cultivation techniques (Zwart *et al.*, 2002), although it has been suggested that only 1% of microorganisms are culturable (Wang *et al.*, 2007; Madigan *et al.*, 2015). However, culture dependent methods should not be discarded as complementary studies.

The Almendares River constitutes one of the most important surface currents in Havana, Cuba; which presents a high level of contamination due to the constant dumping of wastewater of domestic and industrial origin.

In this ecosystem, around 249 polluting sources have been described, coming from the industrial and urban sector, without treatment or inefficiently treated, critical areas of soil erosion, inadequate watershed management

and deforestation (National Institute of Hydraulic Resources, 2010). Although evaluations of the chemical and microbiological contamination of the river have been carried out (Lima *et al.*, 2005; Arpajón *et al.*, 2011; Romeu *et al.*, 2015), the structure of culturable heterotrophic bacterial community have not been assessment before, which could give an idea of the impact it has suffered this ecosystem as a result of the pollution caused by anthropogenic activity. The present work was aimed to characterize the structure of rapid growth culturable heterotrophic bacterial community in Almendares River.

MATERIALS AND METHODS

Sampling

Samples were taken during February, April, June and October of 2009 in Almendares River, considered the dry (November-April) and rainy (May-October) seasons in Cuba (Table 1). The samples were collected from three sampling stations. For the selection of these stations, the differences in the pollution levels were taken as criteria (Arpajón *et al.*, 2011) . Specimens were collected in the morning and transported in sterile plastic bottles of 2L that were placed in a cooler chilled. They were processed in a shorter period of time at 4 hours.

Table 1. Sampling stations of Almendares River
Tabla 1. *Estaciones de muestreo del Río Almendares*

Sampling stations	Latitude	Longitude
Río Cristal	23°01'59.99" N	82°24'03.77" E
Paila	23°03'23.94" N	82°24'09.75" E
Puente de Hierro	23°07'36.55" N	82°24'40.22" E

Isolation and Identification

For water samples isolation, serial dilutions were prepared from 10^{-1} to 10^{-6} in saline (0.8%) and were plated and spread on nutrient agar the dilution 10^{-5} and 10^{-6} (for stations Paila and Puente de Hierro) and 10^{-3} and 10^{-4} (for Río Cristal station). In all cases, were plated 0.1 mL of the dilutions and made three replicates in each sampling station for dilution. The plates were incubated for 48-72 h at 30°C. After incubation, representative colonies of different morphology present in each plate were isolated and reseeded into new nutrient agar plates until purification. Purified isolates were preserved in 20% glycerol. Pure cultures of each bacteria isolated were identified using the API system and associated software identification (bioMérieux, Lyon, France).

To analyze the frequency of isolation of the genera identified in the sampling stations, the data were checked for normal distribution and homogeneity of variance, through the Kolmogorov-Smirnov test and Cochran-Bartlett respectively. Then the multiple comparison tests of proportions, using the statistical package Tonystat (Sigarroa, 1985) were applied.

Determination of Diversity Indexes

To determine the bacterial community structure from the sampling stations studied in Almendares River were calculated species richness (S*), Simpson (L') and Shannon-Wiener (H') indexes (Edwards *et al.*, 2001). These indexes were calculated by the formulas:

$$(L') = \sum_{i=1}^{s} \frac{n_i(n_i-1)}{N(N-1)} \approx \sum_{i=1}^{s} p_i^2$$

$$(H') = -\sum_{i=1}^{s} (p_i)(\log_e p_i)$$

(n_i=number of species i in the sample, s=number of species in the sample, p_i=proportion of species i in the sample, N=number of individuals in the sample)

The differences in S*, L' and H' were evaluated using the Solow's method (Solow, 1993; Edwards *et al.*, 2001). This method uses a random test as follows: isolates from two samples (A and B) are listed, it is estimated the population parameter chosen and calculate the difference in the parameter for the two samples ($D_{A-B} = H'_A - H'_B$).

Isolates from the two samples are bound, are randomly mixture and split into two groups with the original sample size, the population parameter is recalculated for the two samples and determine the new difference. This random mixture and subsequent distribution and calculation of the difference is repeated 10 000 times and organize the absolute values (D_1 to $D_{10\,000}$).

Finally, to assess whether there were significant differences at a confidence level of 95%, the difference (D_{A-B}) was compared with the absolute values of D_1 to $D_{10\,000}$.

If this difference is greater than or equal to the difference to 97.5%, or less than or equal to the value of the difference 2.5%, then the values for the parameter that is analyzed in the two samples are considered significantly different. For the calculations of S*, L' and H' was used an Excel macro-program (Monte Carlo analysis) that would apply the Solow method as explained above.

RESULTS

Structure of culturable heterotrophic bacterial community

A total of 60 isolates were identified, where 23.3% were Gram-negative and 76.7% were Gram-positive. The phylum *Firmicutes* (53.3%), *Actinobacteria* (23.3%) and *Proteobacteria* (23.3%) were the most abundant in the study period (Table 2).

Within *Proteobacteria*, the *Gammaproteobacteria* class (64.3%) was the most abundant. Taking into consideration the taxonomic classification up to the genus level (Table 2), it was observed that the most abundant genera in the Almendares River were: *Bacillus*, *Arthrobacter* and *Acinetobacter*.

Figure 1 shows the distribution of the different genera identified in the sampling stations of the Almendares River in the period February–October 2009. In the sampling station Paila, *Arthrobacter* was isolated more frequently compared with the frequency of isolation of this genus in Río Cristal and Puente de Hierro stations, found highly significant differences ($p<0,001$). However, *Bacillus* was isolated more frequently in Río Cristal station compared with Paila station ($p<0,01$).

Table 2. Relative abundance of the genera identified in Almendares River
Tabla 2. *Abundancia relativa de los géneros identificados en el río Almendares*

Phylum	Class	Order	Family	Genera	Relative abundance (%)[a]
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*	8.3
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Arthrobacter*	15.0
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	45.0
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	8.3
Proteobacteria	*ß-proteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Alcaligenes*	8.3
Proteobacteria	*γ-proteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	3.3
Proteobacteia	*γ-proteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	10.0
Proteobacteria	*γ-proteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	1.7

[a] To calculate the relative abundance, was took into account the number of isolates from each genera in the period February to October 2009 and was divided by the total of isolates obtained in Almendares River per one hundred.

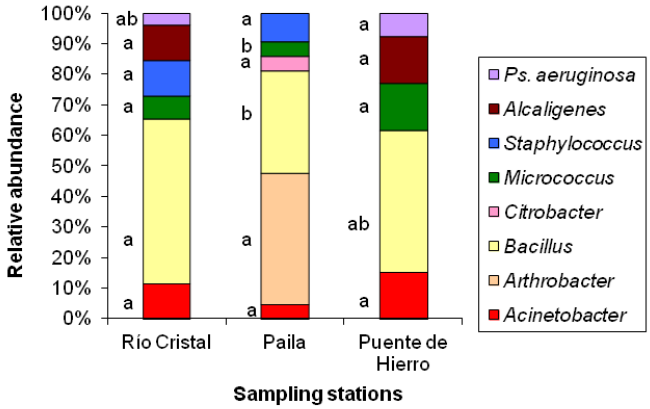

Figure 1. Distribution of the different genera identified in the sampling stations of the Almendares River in the period February–October 2009. Uncommon letters indicate significant differences for Tukey test ($p<0.001$) between the frequency of isolation of the same genus in different sampling stations.
Figura 1. *Distribución de los diferentes géneros identificados en las estaciones de muestreo del río Almendares en el periodo Febrero-Octubre 2009. Letras diferentes indican diferencias significativas según la prueba de Tukey ($p<0,001$) entre las frecuencias de aislamiento de un mismo género en diferentes estaciones de muestreo.*

Regarding species richness, there were no significant differences in the three sampling stations studied (Fig. 2a), but it can be seen that Simpson index (L') (Fig. 2b) and Shannon-Wiener (H') (Fig. 2c) decrease from upstream station (Río Cristal) to downstream station (Puente de Hierro), which means that the probability that two isolates taken at random out to be the same species (expressed through Simpson index) and the bacterial diversity (expressed through Shannon index) decrease in this direction.

DISCUSSION

The composition of the microbiota varies depending on the type of water and mainly depends on the concentration of salts and organic compounds, turbidity, temperature and contaminating sources (Berdjeb *et al.*, 2011; García-Armisen *et al.*, 2014; Wang *et al.*, 2015). In aquatic ecosystems, it is suggested that commonly the bacteria that predominate are Gram negative bacilli -(Ramkumar *et al.*, 2011; Jordaan and Bezuidenhout, 2015). However, in contaminated ecosystems, endospore-forming bacteria have advantages over the rest of the bacteria; since under stress conditions they can form spores. Bacterial spores are resistant structures that contribute to the maintenance and protection of the genetic material of organisms until conditions become appropriate for vegetative growth (Bueche *et al.*, 2013; Mandic-Mulec *et al.*, 2015).

In the present study, Gram positive endospore-forming bacilli predominated among isolates from water samples from the Almendares River. It is an ecosystem contaminated with high concentrations of organic matter with chemical oxygen demand (COD) between 16.7 - 2250 mg.L^{-1} (Larrea *et al.*, 2014).

Taking into account that the members of the *Bacilli* class are Gram positive, aerobic and endospore-forming bacilli (Hikmate *et al.*, 2011) it can be inferred that the majority of the isolates of this research belong to this class. This result shows that the *Firmicutes* phylum is one of the predominant groups in the Almendares River and that in it; the *Bacilli* class is associated with places with a high content of organic compounds such as particles suspended in the water column. Larrea *et al.* (2014) referred that in Almendares River there are high concentrations of total dissolved solids (TDS) with values between 480-4110 mg.L^{-1}.

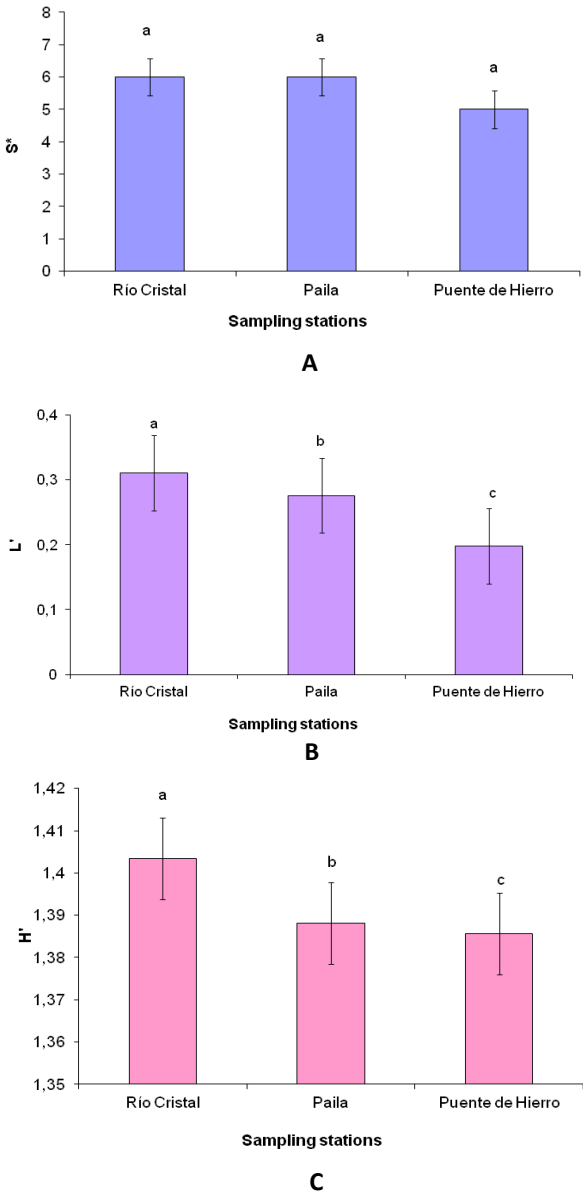

Figure 2. Diversity indexes S* (A), L' (B) and H' (C) at Almendares River stations. Uncommon letters indicate significant differences ($p < 0.05$). The values of diversity indexes were estimated by re-sampling 10 000 times from the original samples and taking the average of the calculated data. Error bars indicate the standard deviation of 10 000 replicates.

Figure 2. *Índices de diversidad S* (A), L' (B) y H' (C) en las estaciones del río Almendares. Letras diferentes indican diferencias significativas (p<0,05). Los valores de los índices de diversidad fueron estimados mediante remuestreo 10 000 veces a partir de las muestras originales y tomando el promedio de los datos calculados. Las barras de error indican la desviación estándar de 10 000 réplicas.*

Reche and Fiuza (2005) and Ramkumar *et al.* (2011), reported that in aquatic systems contaminated with organic matter and with untreated domestic effluents, Gram positive bacteria predominate in water and sediments, and can serve as indicators of contamination by organic matter. Similar results were obtained by Wang *et al.* (2017) who observed that in the polluted Yarlung Tsangpo River, in China, the *Bacilli* class was predominant. Another aspect that could influence the predominance of the *Bacilli* class, is that during the samplings the river flow was very low, and as a result of the resuspension of the sediment, the latter's microorganisms could pass into the water column.

The selective pressure that occurs during cultivation may also have influenced, isolating those cultivable, copiotrophic and fast growing bacteria. In this sense, Burtscher *et al.* (2009) stated that the differences between the culture dependent methods and the independent culture methods, lies in the selective pressure that the culture medium exerts on the microorganisms, taking into account the high concentrations of degradable organic substrates in the $g.L^{-1}$ range, compared to the concentration that can be found in some ecosystems in the range of $mg.L^{-1}$ or $\mu g.L^{-1}$. These are some reasons that justify the fact that through the isolations made in the Almendares River, the *Bacilli* class was more abundant.

In general the predominant genera isolated in the Almendares River were *Bacillus*, *Arthrobacter* and *Acinetobacter*. Most members of the genus *Bacillus* are aerobic heterotrophs and are associated with places with high concentrations of carbon and nitrogen. Furthermore, they play an important role in the processes of denitrification and nitrogen fixation, steps of the nitrogen cycle (Mandic-Mulec *et al.*, 2015).

The *Acinetobacter* genus has been isolated from soil or water samples and has been found frequently in wastewater (Ponce-Terashima *et al.*, 2014). Species of the *Acinetobacter* genus are capable of accumulating phosphates in the form of polyphosphates and may have potentials for the biological removal of phosphates (Doughari *et al.*, 2011). Instead, the genus *Arthrobacter,* can be isolated from aquatic environments where there are high concentrations of ammonium and heavy metals (Kacar, 2015), a situation that occurs primarily in Paila station (Arpajón *et al.*, 2011; Larrea *et al.*, 2014).

The predominant phyla in the Almendares River were *Firmicutes*, *Proteobacteria* and *Actinobacteria*, which is in correspondence with results presented by other authors using both culture-dependent and culture-independent techniques (Zwart *et al.*, 2002; Vaz-Moreira *et al.*, 2011; Read *et al.*, 2015; Herfort *et al.*, 2017; Azmuda *et al.*, 2019). These authors observed that the dominant phyla in bacterial communities of riverine ecosystems are *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* and in some cases *Cyanobacteria*, *Firmicutes* and *Verrucomicrobia*. Particularly, within the phylum *Proteobacteria*, the *Gammaproteobacteria* class predominated, which is typical of contaminated freshwater (Newton *et al.*, 2011; Newton *et al.*, 2013; Newton *et al.*, 2015).

With respect to the bacterial community structure in Almendares River, bacterial diversity decreased from upstream to downstream. Río Cristal station showed the highest values of diversity (H'= 1.40). This station is the less polluted according to Chiroles *et al*. (2007); Arpajón *et al.* (2011) and Larrea *et al*. (2014). Similar results were obtained by Sekiguchi *et al.* (2002), in the Changjiang River, in which bacterial diversity as determined by Shannon index, decreased gradually from upstream to downstream. This result indicated that from upstream to downstream the variety of bacterial species decreased and specific bacterial strains began to dominate.

With regard to the Shannon index of the water samples of Almendares River, is generally lower than that of some other rivers. For example, Xia *et al.* (2014) obtained values of 3.39-4.40 in the Yellow River, and Wang *et al.* (2012), reported values of 1.00-2.40 in the Yangtze River. The Almendares River is a polluted ecosystem, which receives a high pollutant load from factories, industries and the population that lives in the surroundings of its banks (Arpajón *et al.*, 2011; Romeu *et al.*, 2015), all this contributes not only to the poor quality of its waters, but also to the decrease in diversity.

Despite the fact that the Río Cristal station had the highest Simpson and Shannon index values, these are below those obtained in other rivers such as the Yellow River (Xia *et al*., 2014) and the Yangtze River (Wang *et al*., 2012). This may be due to the excessive accumulation of organic matter from aquatic plants and algae present in Río Cristal, which contributes to limiting the diffusion of oxygen and, therefore, influences the structure of the heterotrophic bacterial community.

With regard to Paila station, it was found values of Simpson and Shannon index lower than those in Río Cristal and above Puente de Hierro station.

In previous studies, Arpajón *et al.* (2011) and Romeu *et al.* (2015) argued that Paila was the most polluted station in Almendares River, with high levels of faecal contamination and heavy metals, and low concentrations of dissolved oxygen due to the constant dumping of wastewater from domestic and industrial origin. However, despite being the most polluted station, the lowest values of Simpson and Shannon indexes were obtained in Puente de Hierro station (Figs. 2b and 2c).

Puente de Hierro station is located at Almendares River estuary, with salinity values of 2 439 $mg.L^{-1}$ according to Chiroles *et al.* (2007). It has been reported that tolerance to salinity in brackish water has a selection on the flora and fauna, being poorest in terms of species in estuaries compared to rivers and oceans (Sekiguchi *et al.*, 2002). This could be one of the causes of the low heterotrophic bacterial diversity at this sampling station.

The results of the present investigation have great practical value for the management of ecosystems, since changes in the structure of bacterial communities can be used as indicators of changes in water bodies, which occur as a result of variations in internal processes (ex: physicochemical properties of the water column or retention time) and external processes (ex: climatic conditions, entry of organic matter and nutrients) (Lear *et al.*, 2011; Lear *et al.*, 2012; Washington *et al.*, 2013; Llirós *et al.*, 2014).

Furthermore, these results complement the studies that have been carried out in the Almendares River related to the water quality of this ecosystem. It was observed that the station previously described as less contaminated presented the highest diversity and the stations with the highest contamination presented the least diversity. In this way, through the diversity indices, the impact of pollution on the structure of culturable bacterial communities can be monitored.

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