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RESEARCH ARTICLE

Complexity of river ciliate communities at a national park highlights the need for microbial conservation

Pablo Quintela-Alonso[1] | Blanca Pérez-Uz[1] | Abel Sanchez-Jimenez[2] | Antonio Murciano[2] | Juan D. Centeno[3] | Manuel García-Rodríguez[4] | Esperanza Montero[3] | Benito Muñoz[5] | Cristina Olmedo[5] | Pablo Refoyo[5] | Ismael Velasco-González[1] | Mercedes Martín-Cereceda[1]

[1] Dpto. Microbiología III, F. Ciencias Biológicas, Universidad Complutense de Madrid, Spain

[2] Dpto. Matemática Aplicada (Biomatemática), F. Ciencias Biológicas, Universidad Complutense de Madrid, Spain

[3] Dpto. Geodinámica, F. Ciencias Geológicas, Universidad Complutense de Madrid, Spain

[4] Dpto. Ciencias, Universidad Nacional de Educación a Distancia, Spain

[5] Dpto. Zoología y Antropología Física, F. Ciencias Biológicas, Universidad Complutense de Madrid, Spain

Correspondence
Mercedes Martín-Cereceda, Dpto. Microbiología III, F. Biología, C/ José Antonio Novais 12, Ciudad Universitaria, Universidad Complutense de Madrid, Madrid 28040 Spain.
Email: cerecema@bio.ucm.es

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Abstract

1. Microorganisms play pivotal roles in aquatic ecosystems. Free-living protists are the main components of the eukaryotic microbial communities at the base of freshwater ecosystems. Ciliate grazing channels a large proportion of organic matter into multicellular organisms. Surprisingly, ciliates and other microorganisms are neglected in global conservation schemes.
2. Interstitial ciliates were sampled in three sites of varying human pressure on the River Manzanares (La Pedriza National Park, Spain). Abundances of trophic groups and species were adjusted to a generalized linear model (GLM Poisson regression).
3. Ciliate communities were rich in species (74 morphotypes) and although traditional microscopy retrieved a high number of species that appeared only once or in low numbers, rarefaction analyses estimated much larger species richness. These results illustrate that rarefaction assays are a useful first step for exploring the extent of the ciliate cryptic diversity in freshwater ecosystems.
4. Benthic ciliate communities changed significantly, both spatially and at a short temporal scale. The fluctuating nature of the community was manifested by the presence of many ephemeral species at the same river site, revealing a complex and transient community structure. No significant short-term changes were observed in the physical–chemical properties. Therefore, even slight differences in the abiotic variables may cause rapid shifts of ciliate species.
5. Overall, human pressure had an effect on the interstitial (or benthic) ciliates that resulted in a reduction of species richness and their abundance.

KEYWORDS

benthos, biodiversity, ciliates, generalized linear models, human pressure, microbial habitats, protected areas, protists, river

1 | INTRODUCTION

Surface freshwater habitats support about 6% of all described species despite representing just 0.01% of the world's water (Dudgeon et al., 2006; Gleick, 1996). These ecosystems are the most endangered in the world, and even those that are well protected are at risk from recreational activities and pollution that can reduce habitat quality and biodiversity (Hadwen, Arthington, & Mosisch, 2003; Soller, Bartrand, Ashbolt, Ravenscroft, & Wade, 2010). Freshwater biodiversity provides valuable ecosystem services, such as climate stabilization, drinking water and irrigation, recycling of nutrients, and aesthetic and recreational amenities (Covich et al., 2004; Strayer & Dudgeon, 2010). However, experimental data that show to what extent anthropogenic stressors may affect diversity of

freshwater biological communities are still lacking (Strayer & Dudgeon, 2010).

Spain is one of the countries with the highest biodiversity in the European Union owing to the great variability in climate, orography and geology (Eurostat, 2016). The vast majority of the biodiversity inventories carried out for protected ecosystems in Spain have focused on vertebrates, invertebrates and higher plants. There are no catalogues of microorganisms, except for some diatoms and chlorophyte lists in rivers and ponds (Antón-Garrido, Romo, & Villena, 2013; López-Rodríguez & Rodríguez, 2007). Worldwide biodiversity inventories are also incomplete with respect to the smallest invertebrates (i.e. under 500 μm) and microorganisms. This seems a contradiction when considering the important role of microbial communities in primary productivity, nutrient turnover, predation, degradation, organic matter production and the transfer of energy and carbon to upper trophic levels (Falkowski, Fenchel, & Delong, 2008; Sigee, 2005).

Free-living protists are the main components of the eukaryotic microbial communities at the base of freshwater ecosystems (Caron, 2009; Corliss, 2004; Fenchel, 1987). Their short duplication times and the possession of a simple membrane make the protists ideal for detecting changes in environmental conditions as they respond quickly. Thus, protists are used as effective bioindicators of physical–chemical and nutritional conditions of their habitats (Foissner, 2016; Foissner & Berger, 1996; Payne, 2013). Furthermore, grazing by protists stimulates functional activity of other microbial communities and the rate of organic matter decomposition (Esteban, Finlay, & Clarke, 2012; Fenchel, 1987). Protists increase the turnover of essential nutrients that would otherwise remain locked up in bacterial biomass instead of being channelled to multicellular organisms (Esteban, Finlay, & Warren, 2015). The heterotrophic protists, such as flagellates and ciliates, are the most important consumers of bacteria in freshwater environments (Corliss, 2004; Jürgens & Matz, 2002). Their grazing efficiency over certain bacterial groups (i.e. faecal indicators) may prevent microbial contamination of watercourses and thus improve water quality.

The knowledge of the ecological role of ciliates (and protists in general), combined with the estimation and description of their diversity, is still inadequate despite the recent application of modern molecular tools (Debroas, Hugoni, & Domaizon, 2015; Grossmann et al., 2016; Šlapeta, Moreira, & López-García, 2005). This is largely due to the absence of a single 'species concept' for protists, and has hampered the development of unifying methods for estimating richness (presence) of species and biodiversity (Boenigk, Ereshefsky, Hoef-Emden, Mallet, & Bass, 2012; Schlegel & Meisterfeld, 2003).

Species richness is an important ecological community index that traces potential declines in biodiversity by perturbation of communities (Moritz & Agudo, 2013). However, species richness is very seldom completed by direct counting because rare species may go undetected (Colwell et al., 2012). This is relevant for protists, as rare species have been estimated to comprise 80% of the total pool of species in ciliate communities (Foissner, Agatha, & Berger, 2002). Realistic attempts to inventory species richness of communities and provide future conservation orientated reports on the loss of species require techniques that allow estimating species numbers with confidence (Gotelli & Colwell, 2001). In this sense, rarefaction statistical methods have been proposed as meaningful tools to compare species diversity patterns (Gotelli & Colwell, 2011).

Estimates of species abundance are also required for integrative biodiversity and environmental conservation studies. Obtaining such estimates can be demanding because surveys can be expensive and time-consuming (Potts & Elith, 2006), and more so for the many microscopic and rare protist lineages that need specialized expertise (Debroas et al., 2015). Mathematical models can be very helpful to complement this type of survey work (Leathwick, Elith, & Hastie, 2006; Potts & Elith, 2006). generalized linear models (GLMs) have become increasingly common in ecological studies over the past two decades (Guisan, Edwards, & Hastie, 2002; McCulloch & Neuhaus, 2013). Poisson regression distributions are the most common GLMs used to model count data of populations with a high number of zeros because, for example, many species are rare (either occurring with low abundance or present only occasionally) (Acosta, Zamora, Najarc, Roe, & Hambright, 2015; Lyashevska, Brus, & van der Meer, 2016). However, Poisson regression-GLMs have seldom been applied to the distribution patterns of ciliate communities, either in laboratory microcosms or in natural ecosystems (Clements et al., 2013).

A literature search in the database Web of Science (Figure 1) shows that for the last 15 years there has been a clear increase in research on the biodiversity of microbial communities in general, but still far from satisfactory for protistan communities because they are not included in programmes on species conservation. Recent research on protist geographical distribution suggests that at least one third of protists may have restricted distributions (Bass, Richards, Matthai, Marsh, & Cavalier-Smith, 2007; Foissner, 2008). Therefore, these should be considered for inclusion in IUCN Red Lists for Threatened Species if their habitat is endangered or contaminated. To our knowledge, only one conservation intervention has directly focused on protist diversity (Cotterill, Augustin, Medicus, & Foissner, 2013). The gap between freshwater ecology and conservation biology is still large for protist communities, and more studies are needed to compare protist diversity and community structure in different habitats.

The present work is part of a multidisciplinary research project undertaken in a granite mountain area in Central Spain, recently declared a National Park (La Pedriza, Parque Nacional Sierra de Guadarrama, PNSG). The project aims at cataloguing the diversity of protists in a variety of habitats. The PNSG is a range of mountains and diverse ecotypes only 50 km away from the capital of Spain, Madrid. The Park receives the highest number of visitors per year of all the Spanish National Park network (2 989 556 visitors in 2015. http://www.mapama.gob.es/es/red-parques-nacionales/la-red/gestion/visitantes.aspx%20%5bAccessed%2014%20February%202016]), and the official restrictions and protection of this natural area must coexist with recreational and economic interests. The proximity to the capital research centres has led to a deeper understanding of the geology as well as the biodiversity and ecology of plants and animals. However, no comprehensive studies on microbial communities from this protected area exist yet, which has hampered far-reaching knowledge on the ecological diversity of the National Park.

The aim of this work was to characterize the diversity of benthic ciliate communities in the main river running through this national park

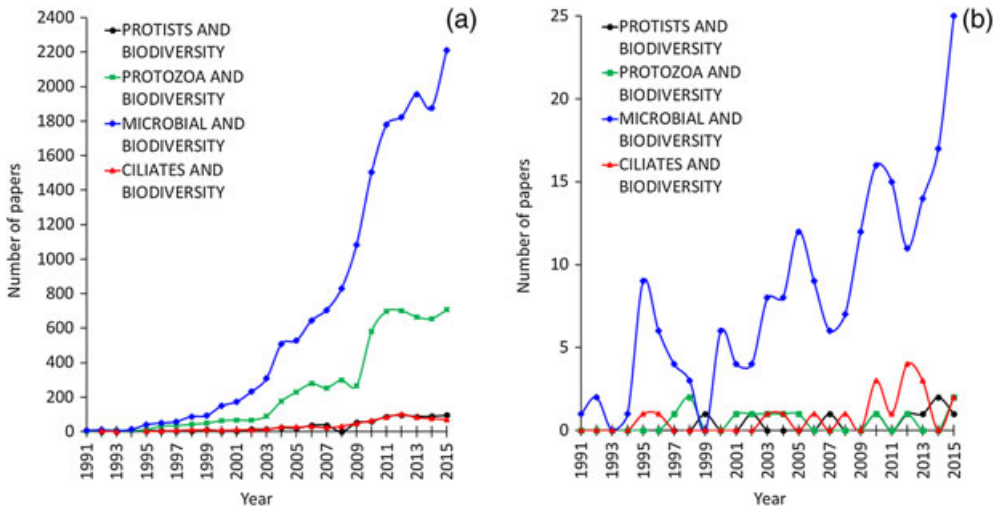

FIGURE 1 Number of manuscripts in web of science database (accessed 10/9/2016) where search terms 'biodiversity' and 'microbial', 'protists', 'protozoa' and 'ciliates' appeared as keywords **(a)** or in the title **(b)** of manuscripts in the last 15 years

as a baseline for future conservation approaches. The study examined the sediments in the upper region of the hyporheic zone of the river. The hyporheic zone serves as a refuge for biological communities in the case of ecosystem perturbation, and it is also, potentially, a place for recolonization of freshwater organisms after temporary droughts. Ciliates are considered to be an important part of the hyporheic community because they have a high reproductive potential and are able to recover from disturbance of sediments through rapid recolonization (Packroff & Zwick, 1996, 1998). However, there is to date much less research on benthic and hyporheic ciliates compared with that on planktonic communities (Cleven, 2004). The present study examined the changes that took place in the structure of the benthic ciliate community after a weekend subjected to high human pressure.

2 | MATERIAL AND METHODS

2.1 | Study area

La Pedriza is a granite landform ecosystem, 3200 ha wide and with an altitude from 800 m to 2100 m. The characteristic landscape consists of massive granitoids of unusual and eye-catching shapes. This area receives thousands of visitors each year who make use of rivers, river banks and water reservoirs potentially contributing to contamination by organic waste and coliform/enterococci bacteria.

La Pedriza is in the upper catchment of the Manzanares River. The source of the river is at the foot of 2200 m peaks, and flows across the National Park southward 92 km across central Madrid City, to its confluence with the Jarama River. As a consequence, water quality is a double concern in La Pedriza, as it affects the water quality of the river as a whole, and also the conservation of a singular landscape and ecosystem.

The three sampling sites in the river are shown in Figure 2:

River Site 1 (RS1) (40°46'17.51"N, 3°54'42.46"O; altitude: 1214 m; mean current velocity: 0.141 m s^{-1}; mean width: 3.2 m; mean depth: 0.53 m; river discharge: 178.9 m^3 s^{-1}) is the most upstream point sampled in the river basin. This site is only accessible for visitors by walking or biking because access to vehicles requires special permission from the Park authorities. The valley surrounding the river is narrow with steep slopes. Sediment grain analysis showed 96.3% sand content, of which 72.6% was in the very coarse and coarse sand fractions (0.5–2 mm diameter) and 18.4% in the medium sand fraction (0.2–0.5 mm diameter). Although there is a significant cover of natural forest, especially of Scots pine (*Pinus sylvestris* L.), the majority (approximately 60%) of the catchment upstream of the site is rocky and steep. As a result, infiltration is low and runoff is high, so that the flow responds quickly to precipitation or ice-melting. This site has very low human impact.

River Site 2 (RS2) (40°45'50.89"N, 3°54'18.03"W, altitude: 1139 m; mean current velocity: 0.167 m s^{-1}; mean width: 2.3 m; mean depth: 0.51 m; river discharge: 276.2 m^3 s^{-1}) is located where there are interconnecting natural ponds and water pools traditionally used by bathers. This is one of the areas of the whole National Park with the greatest human impact; it is also a much visited watering spot for Iberian Ibex (*Capra pyrenaica* Schinz, 1838), which is the most abundant mammal species in the area (Refoyo, Olmedo, & Muñoz, 2016). Here, the valley is wide and the river flows over granitic rock, leaving only small patches of sediment with no riparian vegetation. Sediment grain analysis showed 94.8% sand content, of which 81.2% was in the very coarse and coarse sand fractions and 9.3% in the medium sand fraction. It is an interesting area geologically owing to the dark dykes and the development of torrential erosive landforms on the granitic rock: pools, rills, and polished surfaces.

River Site 3 (RS3) (40°44'56.14"N, 3°53'39.86"W, altitude: 1026 m; mean current velocity: 0.117 m s^{-1}; mean width: 5 m; mean depth: 0.85 m; river discharge: 502.1 m^3 s^{-1}) is the most downstream sampling site. Here the river is wider, deeper and flows slower than in sites 1 and 2; there is a dense riparian vegetation such as shrub formations (*Cistus ladanifer* L., *Crataegus monogyna* Jacq., *Retama monosperma* (L.) Boiss.) and trees (*Alnus glutinosa* (L.) Gaertn., *Quercus faginea* Lam.). Sediment grain analysis showed 97.2% sand content, of which 64.0% was in the very coarse and coarse sand fractions and 27.8% in the medium sand fraction. This site is approximately 200 m

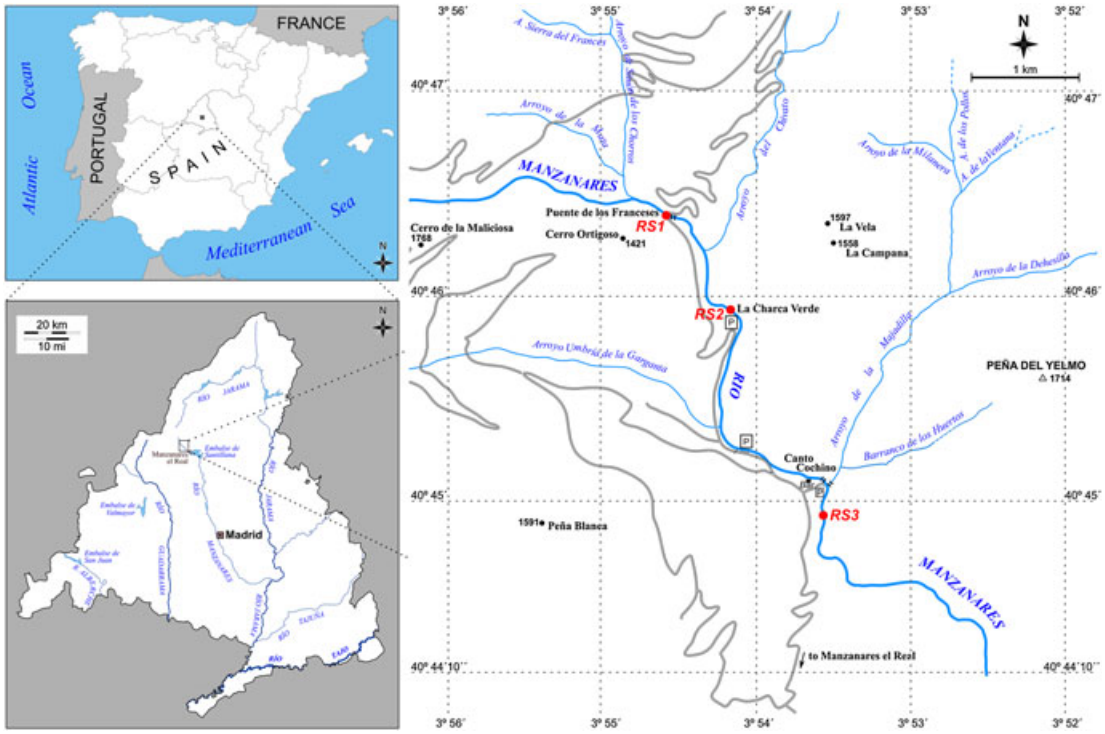

FIGURE 2 Location of sampling sites RS1, RS2 and RS3 in the Manzanares River (La Pedriza, Parque Nacional Sierra de Guadarrama)

downstream from the main parking area and restaurant that opens in the summer season and bank holiday weekends.

At these three sites, samples were collected in May (2015) twice in a week: Monday (day 1) and Thursday (day 2). There were 9200 visitors to the area at the weekend immediately before day 1 sampling, most of whom visited RS2 sampling site. This number was reduced to almost half (4816) on the weekdays before day 2 sampling (PNSG management, pers. comm. March 2017). This sampling schedule was designed to explore the short-term anthropogenic stress from a bank holiday weekend on the river microbial communities.

Sampling of ciliate communities (diversity and trophic groups), bacteriological groups, and physical–chemical parameters, were carried out on both dates at each sampling site.

2.2 | Sampling of ciliates

Water and sediment samples were collected at the three river sites (RS1, RS2, RS3) in shallow areas close to the river bank. At each site, sediment samples were collected using a modified 60 mL syringe with the tip area cut away to fit the internal diameter of approximately 2.8 cm so that it could be used as a corer (total volume: 12.31 cm^3). The syringe was buried 3–5 cm in the sediment and then removed by slowly tilting it and sliding a flat piece of plastic under the syringe to close the opening, to prevent contamination by organisms from the water column. This procedure was repeated three times to obtain a random composite (integrated) sample from a 1 m^2 area of each site. The interstitial water in the syringe was stored in a sterile 100 mL flask by gently tilting the syringe and pushing the plunger. Part of the sediment was then discarded to extract the uppermost 2 cm of river sediment, which was stored in another sterile 100 mL flask. All the samples were stored in portable coolers refrigerated with ice pads until analysis at the laboratory within 8 hours of collection.

2.3 | Abundance and taxonomy of ciliates

For each of the three sampling sites, 2.5 g of the sediment were weighed in a Petri dish (5 cm diameter) and supplemented with 5 mL of the interstitial water extracted from the sediment. The three Petri dishes were kept in a shaker at a low and steady speed to keep the sample mixed during ciliate counts. Live examination is essential to distinguish ciliate morphotypes and ultimately to achieve their correct identification. Therefore, counting and body-size measurement of ciliates from each sample site were conducted under the microscope (at magnifications of 100–1000×) taking at least 10 replicate aliquots of 50 μL of each original sample. These aliquots were covered with a coverslip sealed with Vaseline around the edges to create a chamber preventing the samples from drying during their complete screening at the microscope, and to prevent the specimens bursting because of coverslip pressure. The abundance of ciliates was expressed as the number of ciliates per g of dry weight (DW) of sediment.

Sediment and interstitial water samples were kept in an incubator at 18°C and used in the following weeks to establish cultures and for micrography. The morphological characterization of the species was based on the methods described by Foissner (1991, 2014), i.e. *in vivo* observation using a high-power oil immersion objective and interference contrast, and silver staining techniques (silver nitrate, silver carbonate, protargol techniques).

2.4 | Bacterial analysis

At each of the three sampling sites on both dates, 2 L of water were directly collected by submerging a sterile polyethylene bottle in the

water column following standard protocols. Total aerobic bacteria were determined following the International Standard ISO 6222:1999, total and faecal coliforms by ISO 9308-2, and faecal enterococci by ISO 7899-2:2000 (Corry, Curtis, & Baird, 2011; Da Silva et al., 2013).

2.5 | Physical–chemical analysis

The following physical and chemical parameters were measured *in situ* using a Hanna HI9828 multi-parameter probe: temperature (°C), pH, redox potential (ORP), electrical conductivity (μS cm^{-1}), total dissolved solids (mg L^{-1}), and dissolved oxygen (mg L^{-1}). In addition, at each sampling site 1 L of water was collected with a sterile flask and stored in portable coolers refrigerated with ice pads until analysis in the laboratory. The following water parameters were determined (analytical methods used are indicated in parentheses): bicarbonates (potentiometry), suspended solids (gravimetry), total organic carbon (combustion analysis method and nondispersive infrared detection), Kjeldahl nitrogen (Kjeldahl method), anions – phosphates, nitrites, nitrates, sulphates, chlorides- (ionic chromatography) – and semi-quantitative analysis of metals (inductively coupled plasma-optic emission spectrometry – ICP-OES). Granulometric analysis of the river sediments at the sampling sites was carried out using the Robinson's pipette method (Robinson, 1922). Briefly, this consists in preparing a homogeneous, vigorously stirred soil suspension, and allowing the suspension to settle in a graduated cylinder until all particles greater than a given size have settled 10 cm below the water surface. Then the sample is retrieved with a 20 mL pipette, dried and weighed. The procedure is repeated at standard intervals of time for a range of particle sizes to find the proportion of silts and clay in the sample. Sands are subsequently determined by sieving.

2.6 | Statistical analysis

2.6.1 | Generalized linear models (GLMs)

Poisson regression models were used to evaluate the influence of two explanatory variables or factors – time of sampling (Monday or Thursday) and site of sampling (RS1–RS3) – on the density of ciliates and of bacterial populations in the river. Poisson regressions were chosen because the data comprised counts with a high number of zero values. The initial model (i) included both factors and the interaction between them. A significant interaction meant that the temporal effect on the ciliates depended on the sampling site and vice versa.

$$\text{(i)}\quad \text{Abundance} = e^{\beta_0+\beta_1\cdot s_1+\beta_2 s_2+\beta_3\cdot d_1+\beta_4\cdot s_1\cdot d_1+\beta_5\cdot s_2\cdot d_1}$$

where β_i are the model parameters (i.e. the constant, and the slopes of the factors and their interactions), and s_i and d_i, are the dummy variables (the sampling site and the sampling day, respectively).

$$s_i\begin{cases}1 & \text{if sampling site} = \text{RSi}\\ 0 & \text{in other case}\end{cases} \qquad d_i\begin{cases}1 & \text{if sampling day} = \text{i}\\ 0 & \text{in other case}\end{cases}$$

The modelling strategy was based on a backward stepwise procedure following the hierarchic principle. Owing to the presence of significant interactions between the factors (day and site of sampling), Poisson regression models were applied by selecting one of the factors and using the other factor as a predictor variable. In all cases, the significance of the factors was determined by means of a likelihood ratio test. The analyses were repeated for the abundance of protists grouped by species and functional (trophic) group. The level of significance adopted for all the contrasts was α = 0.05. Linear regression models were used to test the relationship between the physical–chemical parameters and the sampling site locations (represented by the lineal distance between RS1 and the remaining points). Analyses were performed with STATA v. 9.0.

2.6.2 | Rarefaction curves

To estimate the total species richness of the ciliate community at each site and on each day, rarefaction curves were constructed to combine both the number of 50 μL replicate aliquots (replicated incidence data) and the number of individuals (ciliate abundance data) found in the 50 μL replicates. In both cases, the expected number of species in the samples (interpolation) and in augmented samples (extrapolation) was calculated according to Colwell et al. (2012). The asymptotic species richness was estimated by the Chao1 estimator for ciliate abundance data (Chao, 1984) or the Chao2 estimator for replicated incidence data (Chao, 1987). Analyses were performed using custom software written in Matlab© script language.

3 | RESULTS

3.1 | Description of benthic ciliate communities

3.1.1 | Taxonomic assessment and trophic dynamics

All the replicates analysed (N = 63) contained ciliates. Altogether, 74 different morphological species of ciliates (morphotypes) belonging to at least 60 genera and 12 ciliate subclasses (Lynn, 2008) were found at RS1, RS2, and RS3. Figure 3 illustrates some of the diversity of ciliate species found.

Only six of the species (8.1% of the total) occurred in all three sampling sites: *Trochilia minuta* (Roux, 1899) Kahl, 1931, *Cinetochilum margaritaceum* Perty, 1852, *Coleps hirtus* (Müller, 1786) Nitzsch, 1827, *Balladyna* sp. Kowalewski, 1882, *Chilodonella* sp. Strand, 1928, and *Ophryoglena* sp. Ehrenberg, 1831. Moreover, a high percentage of the species were observed only once and with one individual in the replicates analysed (47% at RS1-day 1; 50% at RS1-day 2; 28.6% at RS2-day 1; 36.7% at RS2-day 2; 55.5% RS3-day 1; and 57.1% at RS3-day 2).

The average abundance of ciliates was highest in RS3 (1039.9 per g sediment DW) followed by RS2 (878.6 per g sediment DW) and then RS1, which had a much smaller ciliate population (242.4 per g sediment DW). The most abundant species by site were *Ophryoglena* sp. and *Cinetochilum margaritaceum* at RS1, *Cinetochilum margaritaceum*, *Tachysoma pellionellum* (Müller, 1773) Borror, 1972 and *Trochilia minuta* at RS2, and *Balladyna* sp. *Tachysoma pellionellum* and *Cinetochilum margaritaceum* at RS3. Overall, the most represented subclasses (in presence and abundance) were the Scuticociliatia (scuticociliates) and the Stichotrichia (stichotrichs) (Figure 4).

To examine the pattern of distribution of feeding habits among the ciliates, these were assigned to one of the following functional groups: bacterivores, phytobacterivores (feeding on mixed phytoplankton and bacteria), phytovores (feeding on mixed phytoplankton), carnivores

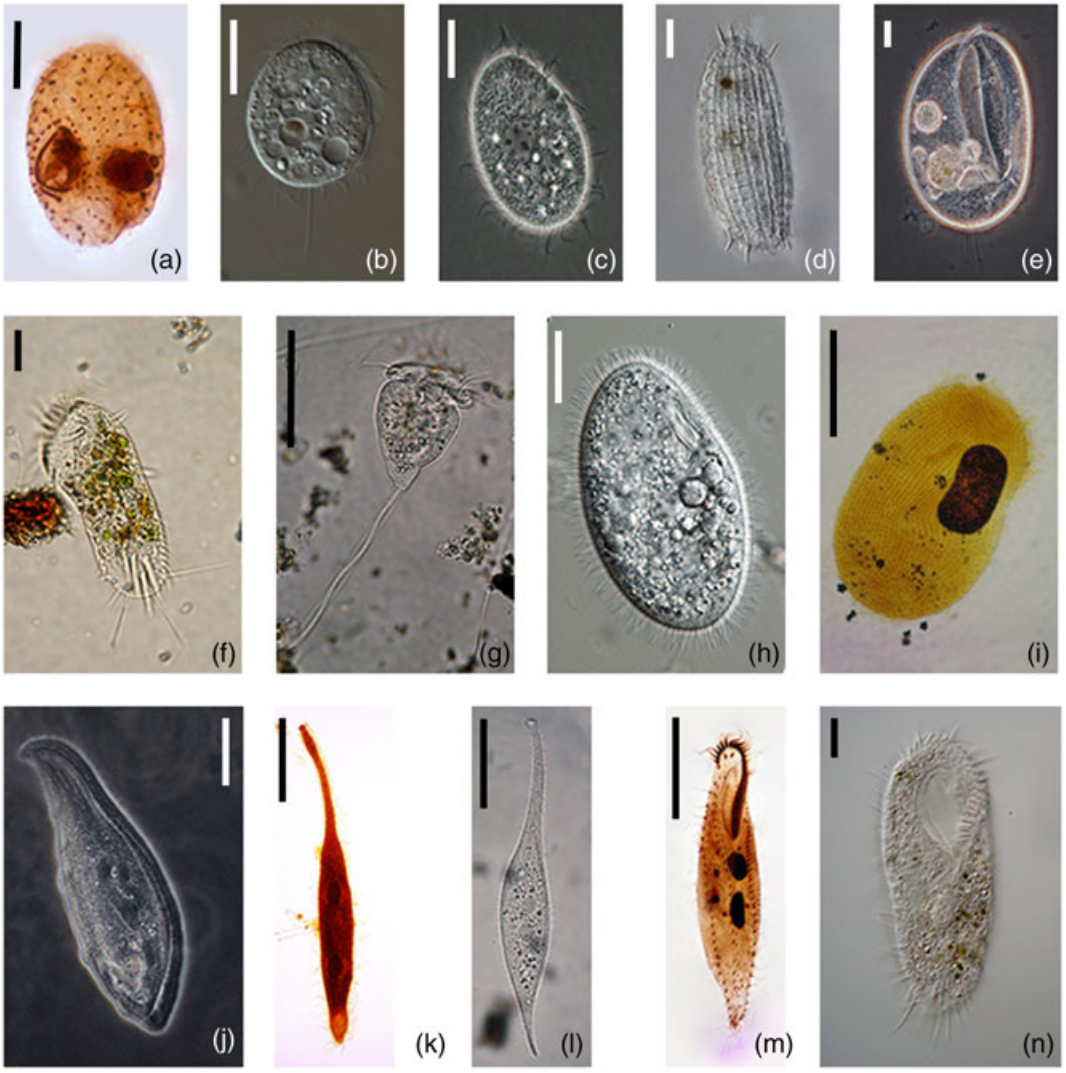

FIGURE 3 Photomicrographs of benthic ciliate species found in the Manzanares River (La Pedriza, Parque Nacional Sierra de Guadarrama). Originals from project MICROEPICS. **(a)** *Cinetochilum margaritaceum* Perty, 1852. Fixed and stained using protargol technique (Foissner, 2014). **(b)** *Cyclidium* sp. Müller 1773. *In vivo* (phase contrast microscopy). **(c)** *Leptopharynx* sp. Mermod, 1914. *In vivo* (phase contrast microscopy). **(d)** *Coleps hirtus* (Müller, 1786) Nitzsch, 1827. *In vivo* (phase contrast microscopy). **(e)** *Lembadion* sp. Perty, 1849. *In vivo* (phase contrast microscopy). **(f)** *Stylonichia mytilus* (Müller, 1773) Ehrenberg, 1830. *In vivo* (bright field microscopy). **(g)** *Vorticella* sp. Linnaeus, 1767. *In vivo* (bright field microscopy). **(h)** *Ophryoglena* sp. Ehrenberg, 1831. *In vivo* (differential interference contrast (DIC) microscopy). **(i)** *Colpidium colpoda* (Losana, 1829) Stein 1860. Fixed and stained with silver carbonate method (slightly modified from Fernández-Galiano, 1994). **(j)** *Loxophyllum meleagris* (Müller, 1773) Dujardin, 1841. *In vivo* (phase contrast microscopy). **(k)** *Trachelophyllum apiculatum* (Perty, 1852) Claparède & Lachmann, 1859. Fixed and stained with Lugol. **(l)** *Litonotus* sp. Wrześniowski, 1870. *In vivo* (bright field microscopy). **(m)** *Uroleptus* sp. Ehrenberg, 1831. Fixed and stained using protargol technique (Foissner, 2014). **(n)** *Steinia* sp. Diesing, 1866. *In vivo* (differential interference contrast (DIC) microscopy). Scale bars = 10 μm **(a–e)** and 40 μm **(f–n)**

and omnivores. The most represented groups across all sampled sites were phytobacterivores and bacterivores, followed by omnivores and carnivores (Figure 5). The phytovores had much lower abundances than any other trophic groups but their abundance was higher at site RS1 than at RS2, and they were not detected at RS3 (Figure 5).

The results on species of ciliates and functional groups showed a variable distribution in space and time. The GLMs applied to trophic groups accounted for a high value of deviance explained in the most representative groups (Table 1). All the models, except for the phytovore model, showed that the interaction between sampling day and site had a significant influence ($P < 0.05$) on the abundance of the ciliate trophic guilds. The exception detected for the phytovore group is probably due to the lack of sufficient data because of their low abundance in the river.

The most representative groups, bacterivores and phytobacterivores, showed generally lower significant values of abundance at RS1 on both sampling days (indicated by the negative value of s_1 slope in Table 2). In the comparison of day 1 versus day 2 for each sampling site (Table 3), RS2 was the site with the highest variability in the structure of functional groups, as all of the trophic groups showed significant differences, and were generally less abundant on day 1 (indicated by the negative value of the d_1 slope in Table 3). This suggests that the higher human pressure on RS2 at the weekend disturbed ciliate populations and reduced their numbers on day 1.

Poisson regression models were also used to examine the human influence by testing the effect of sampling day on the abundance of the most representative species across sampling sites (Table 4). The results showed that the differences were only significant for site RS2

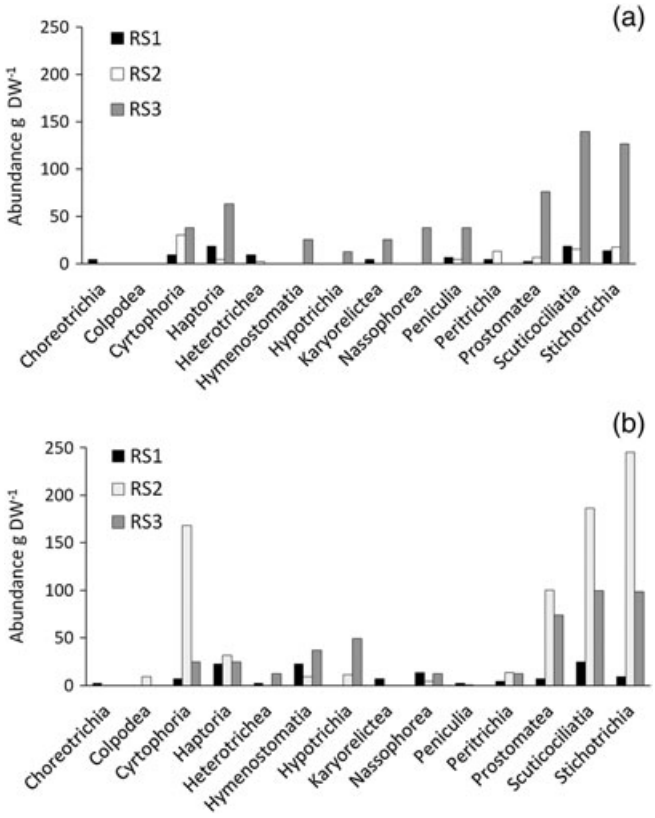

FIGURE 4 Abundance (g DW^{-1}) of taxonomic ciliate subclasses in the river sampling sites (RS1, RS2 and RS3) on day 1 (a) and day 2 (b)

(except for *Ophryoglena* sp. which also showed temporal differences at site RS1), and that in all cases there were always lower densities on day 1 (indicated by the negative value of the d_1 slope in Table 4).

3.1.2 | Comparison of species richness in the ciliate communities

Results from the rarefaction analyses (Table 5, Figure 6) show that the number of species estimated for the three sampling sites (i.e. the asymptote of the accumulation curve) is generally much higher than the number of species observed, except for RS2 day 1, for which both microscopy and rarefaction methods detected a significantly lower number of species than on day 2. At RS1 and RS2 the numbers of observed and estimated species increased from day 1 to day 2, while in RS3 there was a decrease in these numbers. Moreover, independently from the rarefaction method used (individual or sample-based), the difference in the number of species estimated from day 1 to day 2 is consistent at each site. At RS1 the estimated number of species increased from day 1 to day 2 by 17 with both methods; at RS2 the number of species increased by 26 with the individual-based method and by 24 with the sample-based method; while at RS3 the number of species decreased by 9 with both methods (Table 5).

Rarefaction curves were also used to extrapolate the theoretical number of replicates of 50 μL required for analysis at each sample site, in order to reveal the expected number of species that each site harboured. The results showed that sites respond differently, ranging from at least 34 replicates (at RS3) to up to 184 replicates (at RS1) required to assess the species richness of ciliate communities (Figure 7).

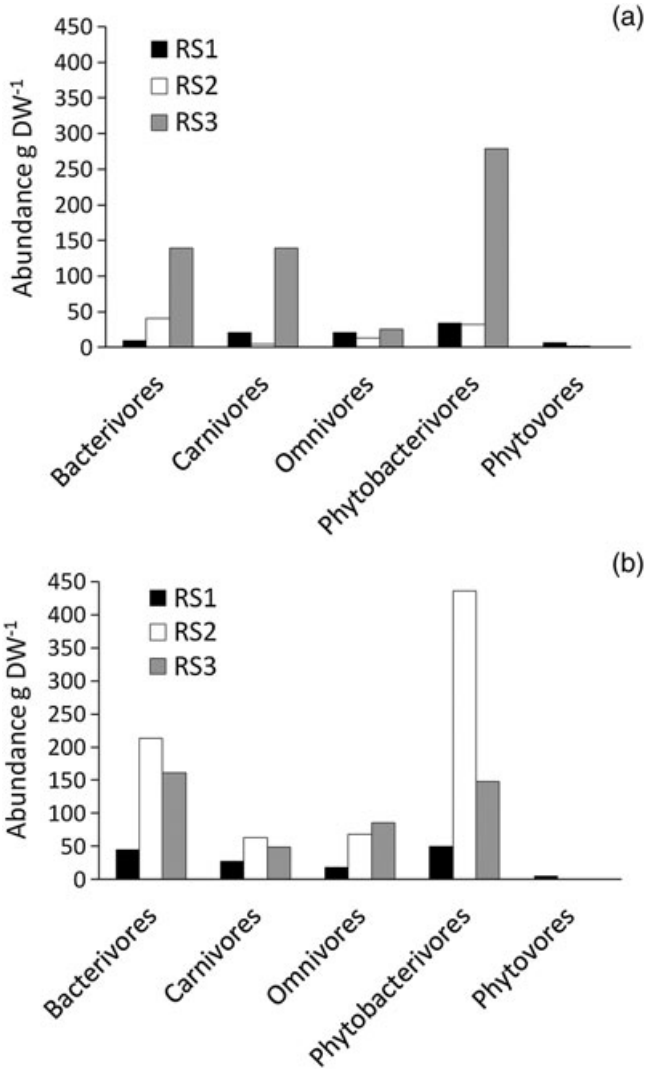

FIGURE 5 Abundance (g DW^{-1}) of ciliate functional groups in the river sampling sites (RS1, RS2 and RS3) on day 1 (a) and day 2 (b)

3.2 | Dynamics of bacterial populations in river water

Poisson regression models for abundances of bacteria in the river are shown in Supporting information Tables S1-S3. The sampling site with the least human influence (RS1) was the location with the lowest bacterial concentration of the three sites on both days (indicated by the negative value of the s_1 slope in the significant models of Table S2). This result coincides with those based on trophic ciliate groups, which showed that RS1 was the site with the lowest abundances of bacterivores and phytobacterivores. The comparison of day 1 versus day 2 for each sampling site (Table S3) shows that at RS1 there were no statistical differences in the concentration of most bacterial groups, probably because RS1 is the most upstream location with hardly any human influence at the weekend. By contrast, at RS2 and RS3, most of the bacterial groups were more abundant on day 1 than on day 2 (indicated by the positive value of the d_1 slope in the significant models of Table S3).

3.3 | Physical–chemical profile of river water

At all sites, the composition of sodium–calcium or calcium–sodium–bicarbonate hydrochemistry facies was consistent with a granitic basin.

TABLE 1 Poisson regression models for the ciliate trophic group abundances in relation to two factors (sampling site and sampling day)

Trophic group	P_M[a]	P_I[b]	Regression model	%D[c]
Bacterivores	<0.0001	0.0090	$A_B = \exp(5.07 - 1.27s_1 + 0.29\ s_2 - 0.14d_1 - 1.45s_1\ d_1 - 1.51\ s_2d_1)$	57.2
Carnivores	<0.0001	<0.0001	$A_C = \exp(3.20 + 0.09\ s_1 + 0.95\ s_2 + 1.42d_1 - 1.68s_1d_1 - 4.10s_2d_1)$	26.7
Omnivores	0.0004	0.0253	$A_B = \exp(4.46 - 1.57s_1 - 0.23s_2 - 1.22d_1 + 1.36s_1d_1 - 0.44\ s_2d_1)$	28.2
Phytobacterivores	<0.0001	<0.0001	$A_{PB} = \exp(4.99 - 1.09s_1 + 1.08\ s_2 + 0.64d_1 - 1.00s_1d_1 - 3.23s_2d_1)$	74.0
Phytovores	0.5258	0.7793	Not significant model	

[a]*P* value of the **m**odel.

[b]*P* value of the factors' **i**nteraction.

[c]percentage deviance explained by the model.

TABLE 2 Site to site comparisons (Poisson regression models) of the ciliate trophic group abundances for each sampling day

		RS1 vs RS2			RS1 vs RS3			RS2 vs RS3		
Trophic group	Day	*P*	β_0	s_1 slope	*P*	β_0	s_1 slope	*P*	β_0	s_2 slope
Bacterivores	1	0.0018	3.72	-1.50	<0.0001	4.94	-2.72	0.0028	4.94	-1.22
	2	<0.0001	5.36	-1.56	0.0009	5.07	-1.27	0.3442^{NS}	5.07	0.29
Carnivores	1	0.0218	1.46	1.57	0.0021	4.62	-1.59	<0.0001	4.62	-3.15
	2	0.0297	4.15	-0.86	0.9044^{NS}	3.20	0.09	0.1590^{NS}	3.20	0.95
Omnivores	1	0.3705^{NS}	2.56	0.47	0.7993^{NS}	3.23	-0.20	0.4398^{NS}	3.23	-0.67
	2	0.0017	4.22	-1.33	0.0044	4.46	-1.57	0.6140^{NS}	4.46	-0.23
Phytobacterivores	1	0.8667^{NS}	3.48	0.06	<0.0001	5.63	-2.09	<0.0001	5.63	-2.15
	2	<0.0001	6.08	-2.18	0.0045	4.99	-1.09	0.0001	4.99	1.08

[NS]Not statistically significant models. β_0: Constant.

TABLE 3 Day to day comparisons (Poisson regression models) of the ciliate trophic group abundances for each sampling site

	RS1			RS2			RS3		
Trophic group	*P*	β_0	d_1 slope	*P*	β_0	d_1 slope	*P*	β_0	d_1 slope
Bacterivores	0.0008	3.80	-1.59	<0.0001	5.36	-1.65	0.7375^{NS}	5.07	-0.14
Carnivores	0.5477^{NS}	3.29	-0.26	<0.0001	4.15	-2.69	0.0445	3.20	1.42
Omnivores	0.7705^{NS}	2.89	0.14	0.0002	4.22	-1.66	0.0946^{NS}	4.46	-1.22
Phytobacterivores	0.2793^{NS}	3.90	-0.36	<0.0001	6.08	-2.60	0.0696^{NS}	4.50	0.64

[NS]Not statistically significant models. β_0: Constant.

TABLE 4 Day to day comparisons (Poisson regression models) of the most representative ciliate species abundances for each sampling site. RS1 = site 1, RS2 = site 2 and RS3 = site 3

	RS1			RS2			RS3		
Species	*P*	β_0	d_1 slope	*P*	β_0	d_1 slope	*P*	β_0	d_1 slope
Trochilia minuta	0.6729^{NS}	1.91	-0.38	<0.0001	4.77	-2.05	0.9831^{NS}	2.51	0.03
Cinetochilum margaritaceum	0.9644^{NS}	2.75	0.02	<0.0001	5.04	-2.48	0.3894^{NS}	3.20	0.72
Coleps hirtus	0.3145^{NS}	1.91	-1.07	<0.0001	4.22	-2.35	0.2340^{NS}	-14.24	16.78
Balladyna sp.	0.9865^{NS}	0.81	0.02	0.0026	2.90	-17.86	0.4514^{NS}	3.61	0.54
Chilodonella sp.	0.0988^{NS}	1.50	-16.73	0.0026	2.90	-17.86	0.0923^{NS}	-13.55	16.78
Ophryoglena sp.	0.0009	2.89	-18.60	0.0334	2.21	-17.86	0.9761^{NS}	3.20	0.03
Tachysoma sp.	No data			<0.0001	4.98	-2.60	0.2655	4.12	-0.89

[NS]Not statistically significant models. β_0: Constant.

The river water is generally characterized by relatively acidic waters, oxidizing conditions, rare mineralization which increases down the river, and low (or, in some cases, non-detectable) amounts of ions, metals and suspended solids (Supporting information Table S4). One sample on day 1 at site RS2 exhibited chlorine concentration possibly related to human presence.

Linear regression models for values of physical–chemical parameters are shown in Supporting information Table S5. Most of the inorganic components increased their concentrations from RS1 to RS3; this reflects the mineralization process occurring as the ions dissolve along the river. By contrast, the organic component did not show statistical differences among the three sites studied in the river.

TABLE 5 Number of observed ciliate species (S_{ob}) and estimated asymptotic species richness (S_{es}) of each site–day assemblage. S_{es} was calculated for both abundance data ($S_{es\text{-}A}$) and replicated incidence data ($S_{es\text{-}I}$)

Sampling site	Day 1			Day 2		
	S_{ob}	$S_{es\text{-}A}$	$S_{es\text{-}I}$	S_{ob}	$S_{es\text{-}A}$	$S_{es\text{-}I}$
RS1	21	34.5	32.4	30	51.4	49.1
RS2	14	17.5	19.5	30	43.7	43.9
RS3	27	46	42.8	21	37.1	34.5

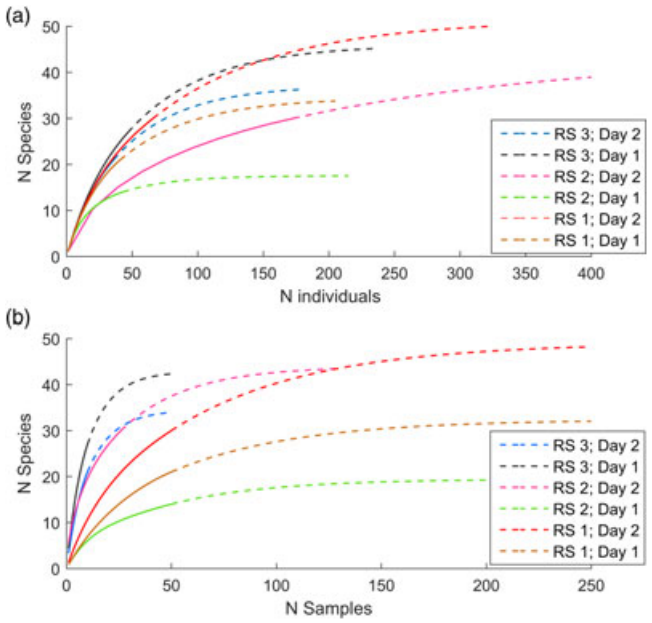

FIGURE 6 Rarefaction curves for each assemblage (site–day) with respect both to the number of individuals (a, ciliate abundance data) and the number of samples (b, replicated incidence data). Interpolation (continuous lines) and extrapolation (dashed lines) data are in both graphs

4 | DISCUSSION

4.1 | General structure of freshwater benthic ciliate communities

A large number of ciliate species (74 morphotypes) were recorded in 2 days of sampling riverine benthic habitats at a National Park in Central Spain. These results broadly fall within the range of 22–170 species reported by previous studies (Andrushchyshyn, Wilson, & Williams, 2007; Dias, Wieloch, & D'Agosto, 2008 respectively; Supporting information Table S6). In the present study, only seven of the species (8% of the total) were common to the three locations sampled, and a high proportion of the species (ranging from 29% to 57% depending on the river site) were found only once in the study and with only one individual. These results are somewhat similar to those reported by Madoni and Bassanini (1999), Madoni and Braghiroli (2007) and Tirjaková and Vďačný (2013), although the proportions of the species found only once in this study were generally higher than previously recorded.

The abundance of ciliates varies greatly in the scale of values reported in the literature depending on the spatial or timescale of the study, as well as on the stream order (Table S6 in Supporting information). The results of this study (Min. 42.8 ind. mL^{-1} at RS1 to Max. 166 ind mL^{-1} at RS3) are in line with average values reported by most studies except Packroff and Zwick (1998) and Tirjaková and Vďačný (2013), who both reported abundances more than one to two orders of magnitude higher. The results reported here show a higher abundance of ciliates per gram dry weight (DW) of sediment, than those found by Königs and Cleven (2007).

Some of these previous works reported the composition of ciliates at coarse taxonomic levels (Cleven, 2004; Madoni & Bassanini, 1999; Madoni & Braghiroli, 2007; Rossi et al., 2016). Scuticociliates and hypotrichs (stichotrichs) are the most abundant and well represented groups, as was also found in the present study. These groups include ciliates with different strategies for mobility and feeding. While scuticociliates are mainly suspension feeders and easily found in the immediate water channel, stichotrichs are more frequently found crawling onto the sediment grains browsing for food. Risse-Buhl, Felsmann, and Mutz (2014) suggested that colonization of sediments by ciliates may be controlled by their cell shape that provides them with more or less ability to become associated with surfaces. They hypothesized that free-swimming suspension feeders are better represented in stable sediments than in the usually shifting sediments. The scuticociliate *Cinetochilum margaritaceum* occurs with the highest abundance and at every site in this study, which has also been reported by other authors (Cleven, 2004; Königs & Cleven, 2007; Madoni & Bassanini, 1999; Madoni & Braghiroli, 2007). *Cinetochilum margaritaceum* is a eurytopic species with rapid ecological adaptability and wide tolerance limits to environmental changes (Madoni & Braghiroli, 2007), explaining its ubiquity in fresh waters.

4.2 | Estimations of ciliate species richness and hints at their rare biosphere

It is essential to estimate the number of species in order to catalogue communities and document the potential loss of species richness from habitats facing perturbation, as well as proposing measures for conservation. Species richness is a commonly used index of ecological diversity. However, it is also a generally difficult variable to measure accurately by direct observation (Hillebrand, Watermann, Karez, & Berninger, 2001; Mora, Tittensor, Adl, Simpson, & Worm, 2011). Species richness increases non-linearly with the number of individuals encountered, the number of samples collected, or the area sampled; therefore, richness estimation by observation usually underestimates the true species number of a community (Rodríguez-Ramos, Dornelas, Marañon, & Cermeño, 2014). This is one of the main problems that microbial aquatic ecologists face when trying to catalogue species richness. A recent study on protist marine phytoplankton found that 20–45% of the species with low abundances were probably missed by conventional analysis of samples by microscopy, and that the examination of subsamples with a microscope showed significant variability in species composition (Rodríguez-Ramos et al., 2014). In ciliate communities this can represent a real hurdle, as they often contain many rare species (Foissner et al., 2002), and many of the species are missed by conventional microscopy. Using classical microscopy, identification of morphotypes in samples taken from diverse ecosystems requires

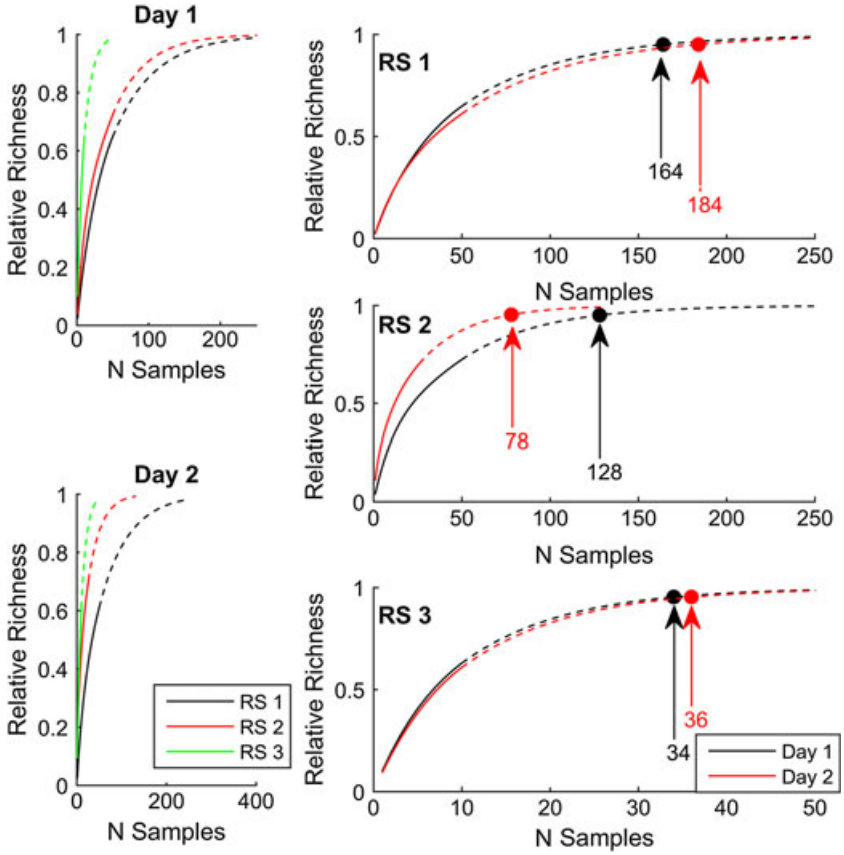

FIGURE 7 Replicated incidence rarefaction for each sampling day (left) and site (right). The number of species was normalized against estimated asymptotic species richness to compare different curves. Points indicate sample effort to get 95% of species of the assemblage

good knowledge and experience of a wide range of microscopic taxa and identification techniques, and nowadays experts in the identification of ciliates are themselves an 'endangered species'. Unfortunately, ordinary molecular approaches to characterize ciliate communities – besides the methodological problems that can arise from the sequence of artefacts – do not really ascertain whether the OTUs (Operational Taxonomic Units) found are active microorganisms or encysted or dormant stages, and therefore if they have a role in the functioning of the ecosystem. Moreover, to assign genetic sequences to signatures of real species does not provide information on the physiology and autoecology of the organism, which is essential for coupling diversity and function under different conditions occurring in nature. At the present time, molecular profiles cannot discriminate the intraspecific variability probably underlying many ciliate genotypes (Caron, 2009; Caron et al., 2009; Santoferrara, Grattepanche, Katz, & McManus, 2016).

In this context, analytical methods such as rarefaction curves can help tools to complement both traditional microscopy and molecular orientated protocols in the exploration of protist biodiversity (Caron, 2009; Dolan & Stoeck, 2011; Rodríguez-Ramos et al., 2014). However, rarefaction inference has seldom been applied by microbial ecologists to investigate the morphological diversity of ciliate communities.

Rarefaction analysis applied in this study to river benthic ciliate communities shows consistently, in both individual and sample number-based approaches, that the estimated species richness is generally much higher than that observed by microscopy. The estimate for the number of species that remain undetected under microscopic observation ranged between 22% at RS2 to 40% at RS1 and RS3 (Table 5 in Results); these percentages are in agreement with the findings of Rodríguez-Ramos et al. (2014) for phytoplankton. In addition, the high number of sample replicates needed to estimate the maximum species capacity of the communities indicates that the sample size was much smaller than the ciliate community size. Rarefaction analysis applied to DNA information (Caron, 2009) has also shown that the curves do not approach an asymptote (the total species richness of the assemblage). Therefore, neither the classical nor the molecular approaches can yet forecast the overall diversity of ciliates. Moreover, the existence of cryptic taxa seems at present to exceed the ability of available microscopy and genetic protocols.

One of the main patterns shown by the freshwater benthic ciliate communities studied here is that a high proportion of the species are rare, occurring only once and with very low abundances. In addition, this study estimated a large proportion of additional species, undetected by microscopy and thus presumably rare. These results coincide with current findings on microbial diversity using molecular approaches, which reveal natural communities dominated by a few abundant species, but also with numerous taxa that occur at extremely low densities. This low-abundance high-diversity pool of microbial taxa was coined the 'rare biosphere' by Sogin et al. (2006). A series of molecular works have confirmed the existence of a large protistan rare biosphere too (Caron, 2009; Caron & Countway, 2009; Debroas et al., 2015; Dunthorn, Stoeck, Clamp, Warren, & Mahe, 2014; Grossmann et al., 2016). It is currently agreed by microbial ecologists that the species richness of most natural protistan communities is still poorly characterized and greatly undervalued (Logares, Mangot, & Massana, 2015).

4.3 | Population dynamics of abundant and rare ciliates

Caron and Countway (2009) visualized the rare biosphere as a continually changing collection of rare taxa rather than a static pool. They proposed that rare species are particularly responsive to minor changes in the environment and may therefore become dominant when environmental conditions change. These periodic shifts of species would confer protist communities with the capacity to buffer the impact of changes. Under this vision, very subtle changes in environmental conditions would cause large and probably very rapid evolution in species composition and community structure. Several studies support this argument. Kazama and Urabe (2016) demonstrated that the abundance and species composition of estuarine tintinnid ciliates varied temporally and changed rapidly within a few days in conjunction with short-term variations of environmental factors. Rossi et al. (2016) determined that the number of morphotypes at time 0 h covered only 57.1% of the diversity present in cumulative checklists after 35 days of sample observation. Countway, Gast, Savai, and Caron (2005) found exceptionally rapid changes in the OTU genetic profile of protist marine pelagic communities from a stable environment. As much as 65% of OTUs were found only once in the three time-periods tested (0 h–24 h–72 h), revealing dynamic communities where taxa rare at a point in time, were capable of becoming important components of the protistan assemblage in a short temporal scale.

The results for freshwater benthic ciliate communities in the present study are similar to those reported by Countway et al. (2005), as the percentage of the species found at only one of the two sampling times (0 h-day 1; 72 h-day 2) was 30% for RS1, 74% for RS2, and 62% for RS3. Thus, benthic freshwater ciliate communities may be at least as temporally dynamic as has been reported by molecular studies for planktonic ciliates. They sustain low-abundant, rare, and even undetected taxa that can only become observed for short periods of time.

The fluctuating behaviour of the ciliate communities studied here is parallel to minor changes in the physical–chemical conditions of the river. In the regression models, the factor 'day' (time) did not show statistical significance for any of the physical–chemical parameters. And although there were statistical differences among the sites for some of the variables, the changes were not abrupt in an ecological scale (Supporting information Tables S4 and S5). Ciliate communities (and bacteria too) were, by contrast, highly changeable and complex in structure and composition of species, as the Poisson models revealed. Trophic ciliate groups and a large proportion of the species had a significantly different ($P < 0.05$) behavioural response in their abundance at some of the river sites in relation to the sampling day and vice versa. Therefore, as Caron and Countway (2009) suggested for pelagic microorganisms, minor changes in environment may have rapid effects on the freshwater benthic ciliate communities too.

Sampling site RS2 deserves particular mention, as it is a densely human populated location during the weekends, so it has more anthropogenic pressure on day 1 (Monday) than on day 2 (Thursday). At this site, large differences in ciliate communities were found between day 1 and day 2. On day 1, RS2 had the lowest global abundance of ciliates and species richness of the river sites (both observed by microscopy and estimated by rarefaction): only 2/5 of the total RS2 species were found on day 1. RS2 was also the site for which the statistical models showed the highest differences in the abundance of trophic groups, the lowest values on day 1. The abundance models for the most representative species in the river also found temporal differences only for RS2, with values of abundance of these species always significantly lower on day 1 as well. Although long-term monitoring is still needed to draw broad conclusions, it seems clear that the high level of human pressure at this location during the weekend affects the structure (species richness and abundance) of ciliate communities. The ways in which this pressure operates are not easy to discern, as there were no harmonizing patterns for changes in the physical–chemical or the bacterial levels at this location. However, clear short-term changes emphasize the sensitivity of ciliates to small fluctuations of abiotic or biotic environmental factors and therefore highlight their importance as indices for bio-indication and biological conservation. Very recently, Grossmann et al. (2016) carried out a broad high-throughput sequencing survey of different types of habitats and discovered that distribution patterns vary strongly between individual taxa of the rare biosphere, suggesting a markedly selective niche adaptation of rare taxa. If these findings are supported by future studies, the rare biosphere may prove a better source for bio-indicator candidates than the most abundant taxa.

4.4 | Conservation strategies for microorganisms and microbial dominated habitats

Nature conservation strategies globally rely upon knowledge of biodiversity and its role in sustainable development. Conservation plans for microorganisms should focus on: (a) incorporating the knowledge acquired in field and laboratory studies within local, national and international biodiversity lists; and (b) establishing the potential economic value of preserving microbial diversity and its contribution to the improvement in the quality of life. The use of repositories for *ex situ* conservation (culture collections of microorganisms, permanent slides and genetic material), and the search for unifying data-mining methods to make information on microbial ecology and taxonomy more user-friendly, are essential first steps for microbial conservation plans.

Some authors have clearly demanded that the value of microbial diversity is incorporated in conservation surveys (Davis & Wilkinson, 2004; Esteban & Finlay, 2010). Griffith (2012) even claimed the need for a Global Strategy for Microbial Conservation (GSMC). However, to our knowledge very few initiatives have been put formally into practice for protist (by extension for microbial) conservation. In July 2016 the International Union for Conservation of Nature (IUCN) evaluated the conservation status of 15 species within the Chromista (protists *sensu lato*), and added four critically endangered species to the Red List. Cotterill et al. (2013) is the only successful case of official conservation of ciliates to date. An ephemeral pond in the town of Salzburg was designated a 'Natural Monument for single-cell organisms'. The conservation arguments used by the authors were: the newly discovered species in the pond (eight new species, five of them endemic to the pond), the preservation of their type locality (for future taxonomic knowledge as a source of reference material), and landscape maintenance.

Disturbance and habitat loss threaten and affect not only plants and animals, but also protists (and other microbes). Because of their size, short generation times and variety of occupied feeding niches, ciliates react faster to a changing environment than higher organisms. Methodological approaches that relate ciliate morphology and DNA sequences in the same study are urgently needed. This would aid implementation of conservation schemes where structure, diversity and function are examined simultaneously. To include ciliate communities that rapidly track changes in the environment will be an exciting challenge for conservation biology. The results of the present study may provide a baseline for future microbial conservation efforts.

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ORCID

Mercedes Martín-Cereceda http://orcid.org/0000-0001-7473-3061

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