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Research Article



Extremophilic cyanobacteria from thermo-mineral springs of spas in Atlantic environments

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The species composition of cyanobacteria assemblages was studied in six thermo-mineral springs of spas in Atlantic environments of Galicia (NW Spain). Two are considered hot ($T^a \geq 40^\circ\text{C}$), two intermediate ($T^a 20\text{--}40^\circ\text{C}$) and two cold ($T^a \leq 20^\circ\text{C}$), and four contain hydrogen sulphide. A total of 21 taxa (14 genera) have been recorded. Two diversity indices, Shannon index and Evenness were determined. The Shannon–Wiener index ranged between 0.31 and 0.73 and the Evenness index between 0.44 and 0.88. nMDS ordination showed that cyanobacteria assemblage composition was influenced mostly by temperature. Of the species identified, the most diverse genus is *Leptolyngbya* with four species, followed by *Chroococcus* with three species and *Aphanocapsa*, *Phormidium* and *Lyngbya* with two species. The most abundant species was *Jaaginema angustissimum*, followed by *Leptolyngbya laminosa* and *Symploca thermalis*. In the two cold springs, seven different species were found, and only *Aphanocapsa conferta* was common to both springs. Cyanobacterial species were more numerous in the four hot springs, with 15 different species and only *Calothrix thermalis* common to these hot springs. It is difficult to establish characteristic cyanobacterial flora for the thermo-mineral waters of the Galician springs since there are significant differences in the communities from the six sites studied.

Key words: Atlantic environments, cyanobacteria, diversity, environmental parameters, Galicia (NW Spain), spa, thermo-mineral springs, thermal-sulphur waters

Introduction

Cyanobacteria are photosynthetic prokaryotic organisms found in very diverse aquatic and terrestrial habitats. In aquatic environments, they develop in marine and fresh water. Within fresh water, we can find them in streams, rivers, ponds, lakes, even in extreme environments such as ice and hot springs. To survive in such varied and sometimes extreme environments, they have a wide range of secondary metabolites, each with specialized functions, to compete successfully in the different habitats of the planet. These secondary metabolites are of great importance from a biotechnological and industrial point of view (Favas et al., 2021; Qamar et al., 2021).

Cyanobacteria are the most ancient oxygenic photoautotrophs and are generally among the dominant primary producers in spring habitats. Springs are extremely diverse ecotones, which can be classified according to different criteria derived from hydrology, geology, hydrochemistry, water temperature, ecology and human use (Glazier, 2009). Various cyanobacterial taxa, which are very diverse depending on environmental conditions, are important in the ecological system of springs, but are also very variable in springs of various types (Cantonati et al., 2012a). Springs are extremely endangered in populated countries by anthropogenic over-use, and the study of the dominating cyanobacterial microflora is important in relation to the control and conservation of water sources (Cantonati et al., 2012b).

According to Cantonati et al. (2015), spring habitats are multiple ecotones (subaerial-submersed, springhead-

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spring stream, groundwater-surface waters). They are of special relevance for biodiversity conservation because of the high diversity they host. The main cause of this high diversity is likely to be the remarkable variety of environmental conditions that the individual springs can represent (from highly shaded to UV exposed, from highly stable, permanent discharge to markedly fluctuating or even intermittent flow, from still water to strong currents, from extremely soft to petrifying water, etc.).

Springs are very heterogeneous not only at the scale of habitat but also at the scale of microhabitat. Within a spring, different substrata are found, and the most common ones are lithic material, bryophytes, surface sediment and organic debris. Likewise, different temperatures are recorded within a spring. Therefore, springs are often said to be microhabitat mosaics (Hájková *et al.*, 2011).

The prevailing environmental conditions during the origin of cyanobacteria in the Precambrian included high temperature, poor levels of free oxygen, high concentration of sulphur and lots of reducing gases such as methane, ammonia and carbon dioxide. These extreme environmental conditions are similar to those of present-day thermal springs (Castenholz, 1973).

Highly developed cyanobacteria in microbial mats of geothermal springs are common at temperatures less than 74°C and the pH more than 5.0. *Synechococcus*, *Phormidium*, *Calothrix* and *Mastigocladus* are some of the cyanobacteria taxa found in thermal springs. However, their distribution pattern varies with temperature ranges (Arman *et al.*, 2014).

The hot spring mat communities in terrestrial habitats have long attracted the attention of microbial ecologists because of the unique adaptations of the microbial flora to these extreme environments (Castenholz, 1973). The absence of predators simplifies understanding of ecosystem structure and allows interpretation of species diversity and community structure in terms of temperature gradients (Ward *et al.*, 1998) and salinity gradients within spring systems, or in terms of water chemistry (Skirnisdottir *et al.*, 2000) between spring systems.

Galicia is one of the European regions where the use of mineral and thermal waters has an extensive tradition, as evidenced by the numerous existing vestiges of exploitations that date back to Roman times. In fact, from the first recorded treaty in 1697 until today, there are numerous historical documents in which the existence and use of mineral waters in Galician spas are mentioned (Corral & López, 2010). The regional geological diversity ensures high variability in the hydrochemical facies of its mineral waters, and for this reason many different spring types are found, including the most common, cold and moderately mineralized,

thermal or saline springs. In addition, one characteristic of this area is the presence of many sulphur springs that are formed where deep waters emerge through igneous and metamorphic rocks of high sulphide content, where conditions can be anoxic. Many of these springs are also unpolluted by excessive nitrogen and phosphorus from natural and anthropogenic sources (Leira *et al.*, 2017).

In order to discover the cyanobacterial communities capable of surviving in such extreme environments, a study was done in six representative springs of spas in Galicia, using classical morphological studies with microscopy examination. The sampled springs present great physical and chemical diversity, mainly related to temperature. A multivariate statistical analysis was used to examine the interactions between the cyanobacteria found and the physical and chemical characteristics of the springs.

Materials and methods

Location and description of the springs sampled

Six springs in Galicia (NW Spain) were selected for this study (Fig. 1). Burgas, Outariz, Chavasqueira and Pantón spas are located in Ourense province; Guitiriz spa in A Coruña and Cuntis spa in Pontevedra province. Galicia is located in the so-called wet Iberia with an oceanic climate. Its main features are the regularity of rainfall during the year, from 1000–1500 mm per year, and mild temperatures with low annual oscillation. The selected springs are representative of the broader range of springs that can be found within the Iberian massif. In addition, they are considered unchanged since ancient times and are currently used for balneotherapy. Of these six springs, four are considered thermal springs, Burgas (42°20'04"N, 7°51'55"W), Outariz (42°20'56"N, 7°54'58"W), Cuntis (42°38'10"N, 8°33'45"W) and Chavasqueira (42°20'59"N, 7°52'46"W); and two cold-water springs, Guitiriz (43°10'41"N, 7°53'32"W) and Pantón (42°30'58"N, 7°36'08"W). Three of these springs (Cuntis, Pantón and Guitiriz) are also considered sulphur springs (Leira *et al.*, 2017).

The Burgas, Outariz and Chavasqueira springs are characterized by high temperature (52.6–59.6, 45–49 and 53.5–55°C, respectively) and are located in the city of Ourense, at the bottom of a valley carved by the Miño River. The three springs emerge through a dense network of fissures (Delgado-Outeirino *et al.*, 2009). Lithologically, they are situated on granite crystalline rocks covered by alluvial soils and their waters emerge at the highest temperature of all the thermal springs in

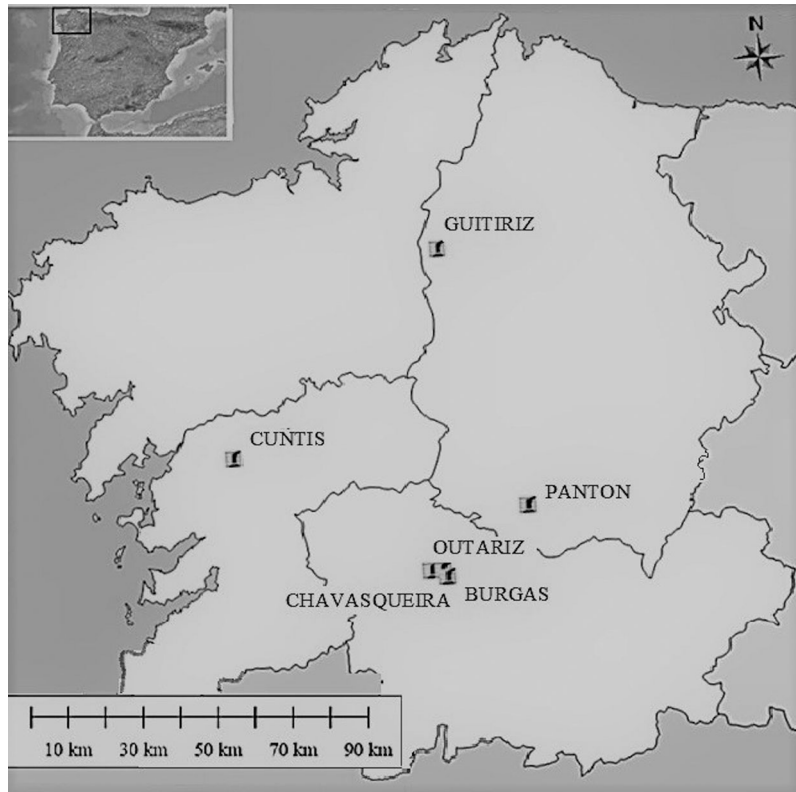


Fig. 1. Map showing the location in Galicia of the sampled springs (the map in the upper left corner is Spain).

Galicia. With a flow rate of 50 L s^{-1} , Burgas flows through a 19th century granite building, comprised of a niche with a central pillar that sits in a 7 cm deep basin. This basin receives the water that overflows from the upper 1 cm deep basin. Some of the water that overflows from the lower basin runs through a narrow channel where the water accumulates. The samples from this spring were taken by scraping the surface of the granite stones (Fig. 2). The Outariz (Fig. 3) and Chavasqueira (Fig. 4) springs emerge directly among rocks at the edge of the Miño River with a flow rate of 8 and 2 L s^{-1} respectively, running through the rocks down to the river. The samples were taken from the rock surfaces.

Guitiriz and Pantón springs are considered cold ($16\text{--}17.3$ and $19\text{--}20^\circ\text{C}$, respectively) and are located in the Ollo de Sapo area where there is great rock diversity, in addition to the dominant granite massifs (Corral & López, 2010). Guitiriz is comprised of an artificial stone fountain with a semi-circular basin fed by water from two pipes. The basin is about 90 cm long, 50 wide and 20 deep, and excess water drains into an underground basin. The flow rate is 0.5 L s^{-1} . Samples were taken from the walls of the fountain (Fig. 5). The Pantón spring emerges directly from the rocks with a flow rate of 1.5 L s^{-1} . Its waters are collected in a rectangular basin via two pipes. The water drains

continuously through a hole in the bottom of the basin. Samples were taken from the walls of this basin (Fig. 6).

The Cuntis spring experiences deep hydrothermal flows and intermediate chemical evolution passing through granites and biotitic granodiorites, and its water is considered warm to hot ($48\text{--}54^\circ\text{C}$). Samples from this spring were collected from a granite channel, 20 cm wide, 15 cm deep and 8 m long, through which water flows continuously at 0.6 L s^{-1} . The thermal water of this spring emerges inside a nearby building, passing through a hole in the wall (Fig. 7).

Physical and chemical characteristics

Water temperature, pH and conductivity were measured *in situ* at four different points in each spring, as close as possible to the cyanobacteria sampling sites, with portable instruments calibrated in the field. Temperature and pH were measured with an HI 9014 Hanna pH meter microcomputer and conductivity was measured using an HI 9033 multi-range conductivity meter. Water samples were collected in 1 L polyethylene bottles to allow major ions to be analysed in the laboratory, following standard procedures and methods (Apha, 2005).



Figs 2–7. Sampled springs: Burgas (2), Outariz (3), Chavasqueira (4), Guitiriz (5), Pantón (6), Cuntis (7).

Sampling and identification of cyanobacteria

Four replicates of the benthic microalgal mats on solid substrates were scraped with a sterile spatula and collected in tubes that were maintained in cold, dark conditions until arrival at the laboratory, where they were preserved in formaldehyde (3.7%) to evaluate the biodiversity. Cyanobacteria were studied and identified with a stereomicroscope and a light microscope (LM) (OlympusBX61) with a $\times 100$ oil immersion DIC lens. Light photographs of the most abundant taxa were taken with a DP70 Olympus camera. The main monographs used for taxonomic identification were Geitler (1932), Komárek and Anagnostidis (1999, 2005), Komárek (2013) and Komárek et al. (2014).

Data and statistical analysis

One-way analysis of variance (ANOVA) and the Tukey test were performed to determine significant differences ($P < 0.05$) among springs in physical and chemical variables. These tests were performed in the SPSS statistical package version 26. R package Vegan version 2.5.6 was used in the rest of the statistical techniques. Two diversity indices, Shannon index and Evenness were determined. For the ordination techniques and in order to reduce the distortion of highly abundant species, abundance data were transformed by square root. Non-metric multidimensional scaling (nMDS) analyses of species with the metaMDS function of Vegan were applied to highlight the possible differences among springs. Similarity matrices based on the Bray–Curtis similarity index was used for this analysis. The differences were assessed using a one-way analysis of similarities test (ANOSIM). Similarity percentage analysis (SIMPER) was used for identifying the species that were most important in creating the observed pattern of differences (group separation) in the nMDS. Linear relationships between environmental variables and nMDS scores were evaluated using the *envfit* function of Vegan. Environmental variables were previously transformed with $\log(x + 1)$ to improve normality.

Results

Physical and chemical characteristics of the water of each spring

Eight physical and chemical parameters determined for each spring are shown in Table 1. There was great variability especially in temperature, conductivity and pH. Figure 8 shows boxplots of these parameters that allowed characterizing the water of each spring.

ANOVA was able to detect significant differences among the springs in all of the measured variables. Conductivity, H_2S concentration and temperature were the variables with the greatest significant differences, while the calcium and SO_4 concentrations showed the smallest differences.

With respect to temperature, the Tukey test grouped the springs into three groups; Burgas and Chavasqueira in the high temperature group, Pantón and Guitiriz in the low temperature group, and an intermediate group (although closer to the high temperature group) comprised of Outariz and Cuntis. Therefore, two springs, Burgas and Chavasqueira, can be considered hot ($T^a \geq 40^\circ\text{C}$); two, Outariz and Cuntis, intermediate ($T^a 20\text{--}40^\circ\text{C}$); and two, Pantón and Guitiriz, cold ($T^a \leq 20^\circ\text{C}$).

The pH of all springs was above 7.0 (weakly alkaline or alkaline). The most alkaline spring was Guitiriz with a pH of 9.5 ± 0.2 . In addition, Guitiriz was the spring that presented a higher iron concentration ($302.5 \pm 17.1 \text{ mg L}^{-1}$).

Burgas was the spring that presented the highest conductivity with $866 \pm 21 \text{ }\mu\text{S cm}^{-1}$, while Pantón and Guitiriz were the ones that had the lowest, not presenting significant differences between them and with an average conductivity of $344 \text{ }\mu\text{S cm}^{-1}$. Nor were there significant differences in the conductivity of Chavasqueira and Cuntis, with an average conductivity of $526 \text{ }\mu\text{S cm}^{-1}$.

Four springs presented sulphurous waters, especially Cuntis, which presented the highest content of H_2S ($7.4 \pm 0.5 \text{ mg L}^{-1}$). In addition, Cuntis was characterized by having a higher chloride ($56.8 \pm 1.7 \text{ mg L}^{-1}$) and sulphate content ($45.9 \pm 1.1 \text{ mg L}^{-1}$).

With regard to calcium, Pantón presented a higher content ($5.3 \pm 1.3 \text{ mg L}^{-1}$).

Cyanobacterial species composition

Twenty-one different species of cyanobacteria were found in these springs (Table 2). The most abundant species were *Jaaginema angustissimum*, notable for its presence in Guitiriz (cold spring), and *Leptolyngbya laminosa*, mainly in Outariz (hot spring). In the two cold springs, seven different species were found, and only *Aphanocapsa conferta* was common to both springs. Cyanobacterial diversity was higher in the four hot springs, with 15 different species (nine in the highest temperature springs and six in the intermediate), and only *Calothrix thermalis* was common to these hot springs, both at high and intermediate temperatures.

Among the most significant species are the following:

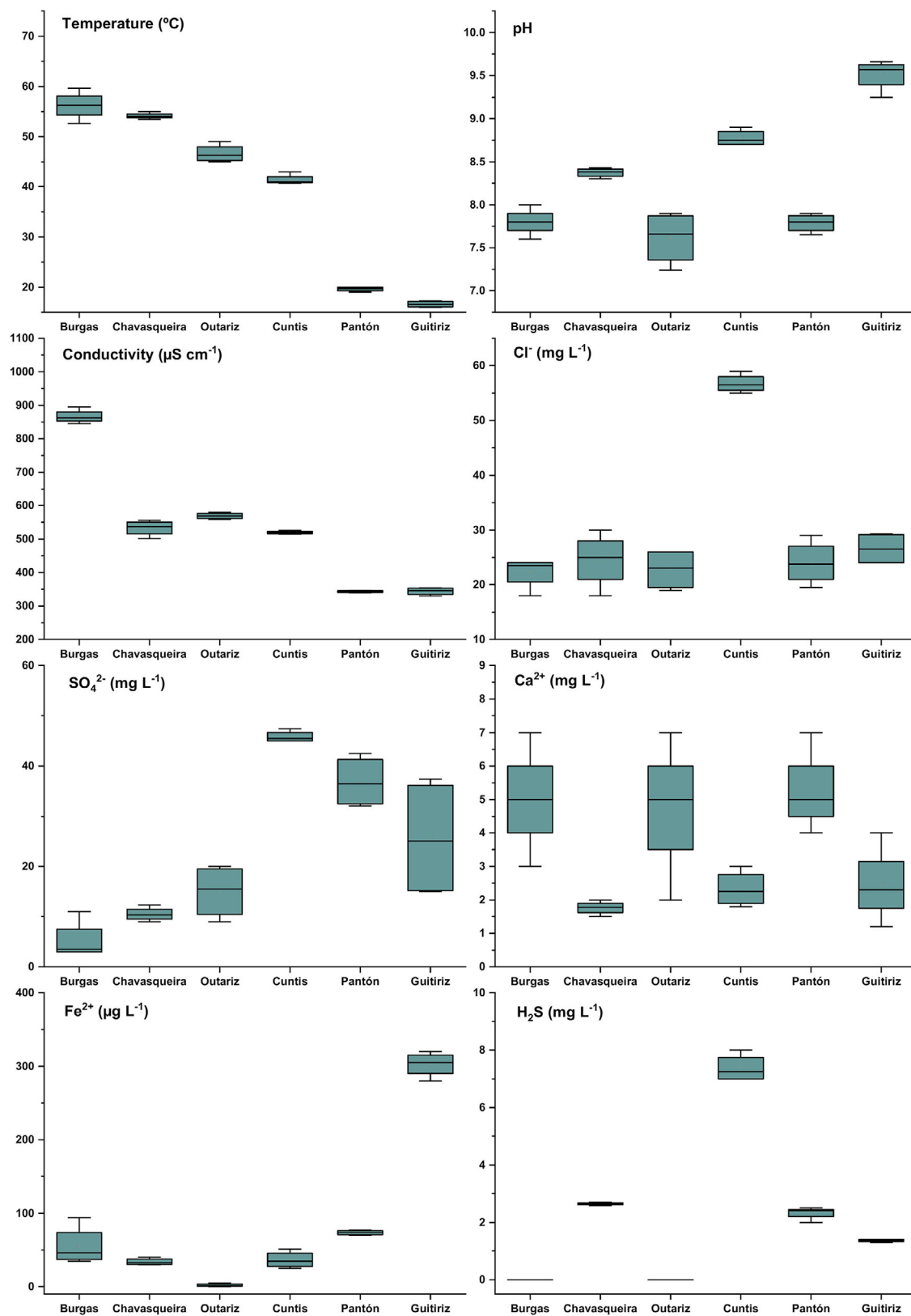
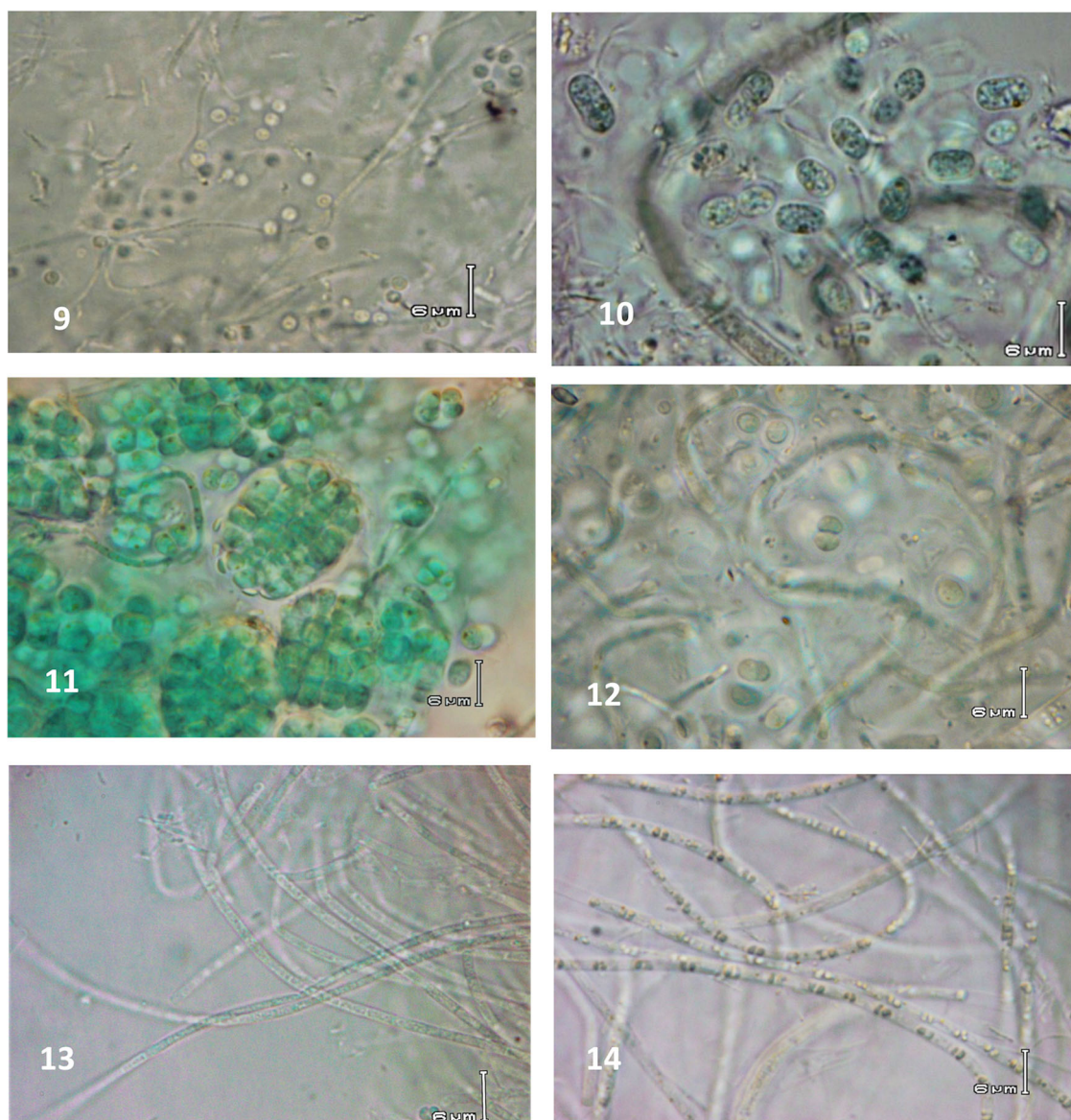


Fig. 8. Boxplots of the eight physical and chemical characteristics of the six springs obtained at the sampling points ($n=4$).



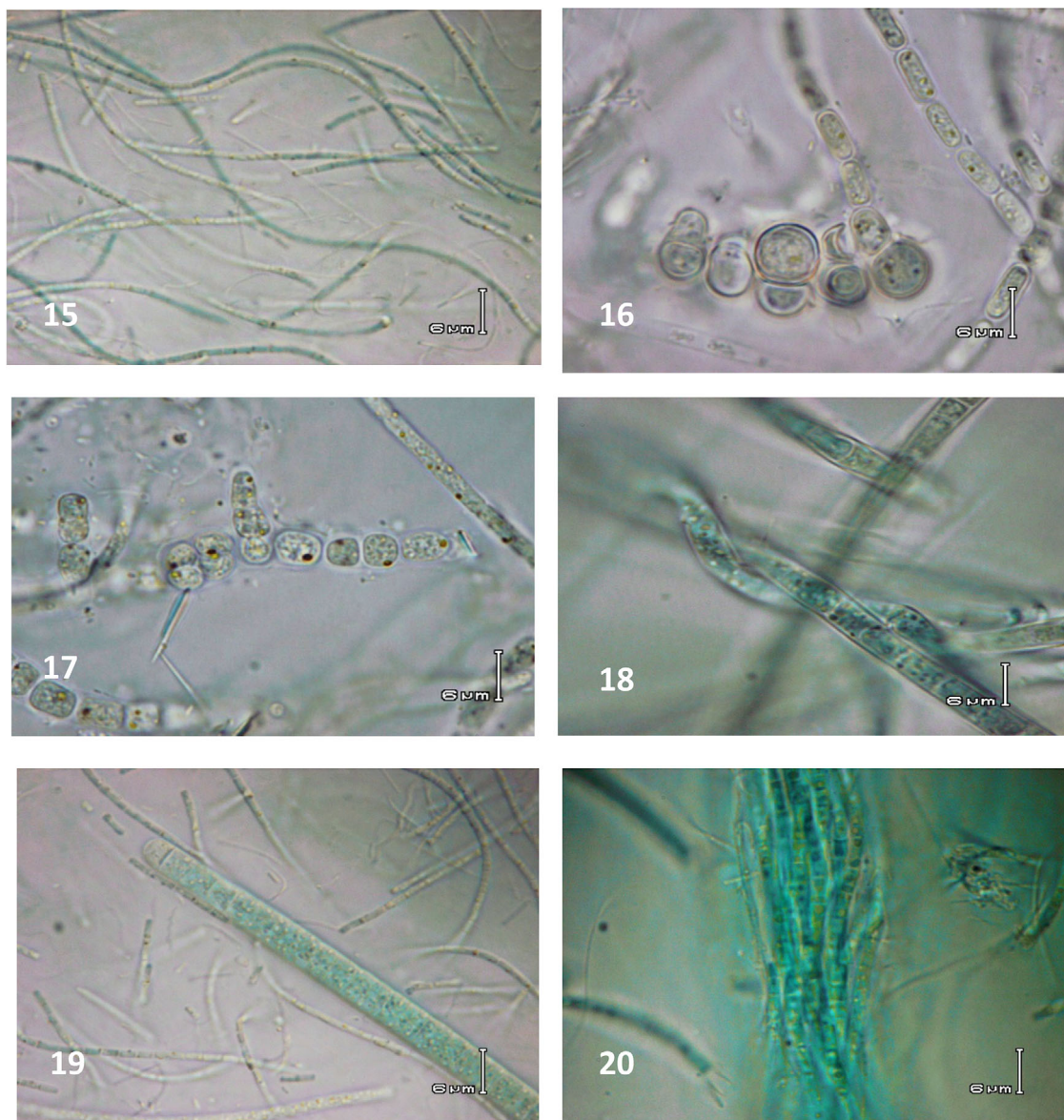
Figs 9–14. LM micrographs of *Aphanocapsa thermalis* (9), *Aphanothece bullosa* (10), *Chroococciopsis thermalis* (11), *Chroococcus thermalis* (12), *Jaaginema angustissimum* (13), *Leptolyngbya granulifera* (14).

Table 1. Physical and chemical properties of the water of the springs sampled in Galicia.

	As Burgas	Chavasqueira	Outariz	Cuntis	Pantón	Guitiriz
Temperature (°C)	56.2±2.9	54.1±0.6	46.6±1.8	41.4±1.1	19.6±0.5	16.6±0.6
pH	7.8±0.2	8.4±0.1	7.6±0.3	8.8±0.1	7.8±0.1	9.5±0.2
Conductivity (μS cm ⁻¹)	866±21	533±24	569±9	520±5	343±3	344±11
Cl ⁻ (mg L ⁻¹)	22.3±2.9	24.5±5.0	22.8±3.8	56.8±1.7	24.0±4.0	26.6±3.0
SO ₄ ²⁻ (mg L ⁻¹)	5.3±3.9	10.5±1.4	15.0±5.4	45.9±1.1	36.9±5.2	25.7±12.2
Ca ²⁺ (mg L ⁻¹)	5.0±1.6	1.8±0.2	4.8±2.1	2.3±0.5	5.3±1.3	2.5±1.2
Fe ²⁺ (μg L ⁻¹)	55.3±27.0	34.0±4.5	2.0±2.2	36.5±11.5	73.5±3.1	302.5±17.1
H ₂ S (gas, mg L ⁻¹)	n.d. ^a	2.6±0.1	n.d. ^a	7.4±0.5	2.3±0.2	1.4±0.1

Aphanocapsa thermalis (Fig. 9). Spherical blue-green cells, 1.2–2 μm in diameter, grouped forming irregular colonies with colourless mucilage. Common in thermal

springs (up to 68.7 °C) of the northern hemisphere, it is recorded in Greece and Slovakia (<http://www.algaebase.org>; 27 May 2020).



Figs 15–20. LM micrographs of *Leptolyngbya laminosa* (15), *Mastigocladus laminosus* (16, 17), *Phormidium boryanum* (18), *Phormidium lucidum* (19), *Symploca thermalis* (20).

Aphanothece bullosa (Fig. 10). Oval cells or elongated cylindrical cells with rounded ends. Finely granular content and pale blue-green, $3\text{--}4 \times 6\text{--}7\ \mu\text{m}$. Colonies irregular with colourless mucilage. Recorded in thermal waters ($20\text{--}50\ ^\circ\text{C}$) from Greece, Italy, Slovakia (Komárek & Anagnostidis, 1999) and Spain (Alvarez-Cobelas & Gallardo, 1988) in Europe; also in Africa, Asia and Central America (Komárek & Anagnostidis, 1999).

Chroococidiopsis thermalis (Fig. 11). Spherical or irregular colonies with blue-green cells up to $5\ \mu\text{m}$

surrounded by thin colourless envelopes. Colonies of varied size: small 4-celled young colonies added in tetrads, up to colonies $15\ \mu\text{m}$ in diameter. Known mainly in tropical countries, less frequently in temperate zones such as Europe, where it has been recorded in Germany, Greece and Slovakia (Komárek & Anagnostidis, 1999).

Chroococcus thermalis (Fig. 12). Few celled, usually 2–8-celled colonies. Colourless mucilaginous envelopes. Cells dull blue-green, $4\text{--}5\ \mu\text{m}$ in diameter. Recorded in thermal waters (up to $36.6\ ^\circ\text{C}$) in Europe, Japan and North America (Komárek & Anagnostidis, 1999).

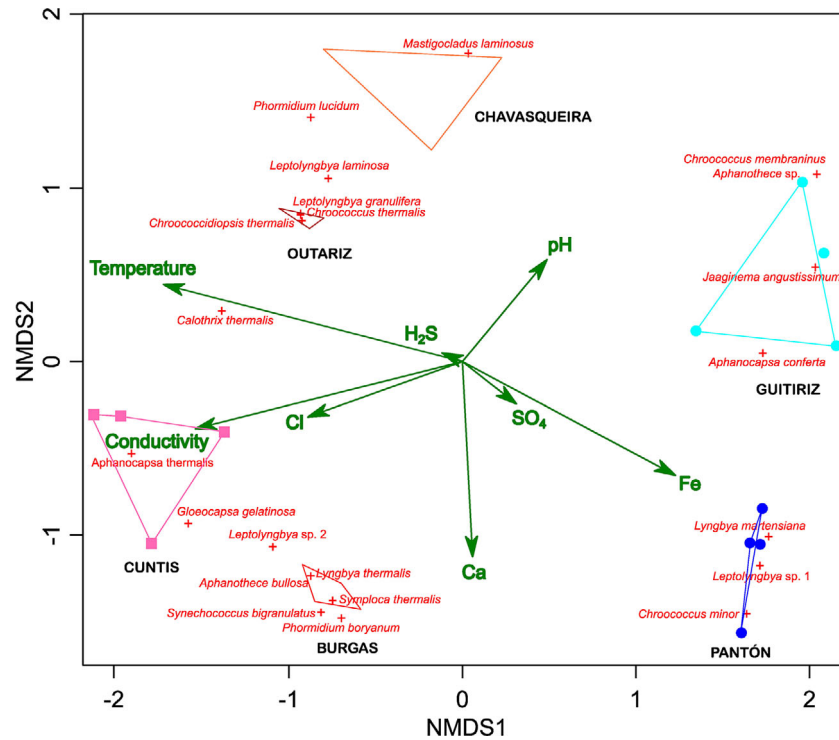


Fig. 21. Non-metric multidimensional scaling (nMDS) ordination of the springs based on the cyanobacterial species composition found in them. The fitted vectors of the eight environmental variables are also represented (green lines with arrows).

***Jaeginema angustissimum* (Fig. 13).** Pale blue-green trichomes, not constricted and ungranulated cross-walls, ends not attenuated and apical cell rounded. Cells $3.6\text{--}4.8 \times 1.2\text{--}1.6 \mu\text{m}$. Recorded in thermal and sulphur springs in Spain (Gonzalez Guerrero, 1944), Austria, England, Georgia, Greece, Hungary and outside of Europe in North America, Asia and South Africa (Komárek & Anagnostidis, 2005)

***Leptolyngbya granulifera* (Fig. 14).** Flexuous to contorted filaments. Sheath is thin, colourless, almost invisible. Blue-green cells $4\text{--}6 \mu\text{m}$ long \times $2\text{--}2.2 \mu\text{m}$ wide, with a granule on either side of the cross-walls.

Recorded in thermal springs in Europe (Greece), USA, India and Japan. <http://www.algaebase.org>; 1 June 2020.

***Leptolyngbya laminosa* (Fig. 15).** Filaments almost straight or curved, or densely entangled. Sheaths colourless and very thin. Trichomes pale blue-green. Cells longer than wide: $2.4\text{--}6 \times 0.8\text{--}1.2 \mu\text{m}$, usually with a single granule on either side of the cross-walls. Occurrence in thermal springs usually accompanying *Mastigocladus laminosus*, distributed worldwide (Komárek & Anagnostidis, 2005).

***Mastigocladus laminosus* (Figs 16, 17).** Filaments densely entangled, true branched (by T-, V- or rarely Y-shaped branching). Branches usually narrower than main filaments. Trichomes constricted in old parts, cylindrical and unconstricted in branches. Cells barrel-shaped, $4 \mu\text{m}$ diameter, often in irregular length in main filaments, cylindrical in branches and often distinctly longer than wide. Intercalary heterocytes, spherical up to $6 \mu\text{m}$. Recorded as cosmopolitan in thermal springs around the world (Komárek, 2013).

***Phormidium boryanum* (Fig. 18).** Trichomes solitary, dark greyish blue-green and irregularly, loosely coiled in a screw-like manner, finely granulated cross-walls and gradually attenuated at the ends. Most cells nearly isodiametric $3\text{--}4 \mu\text{m}$, with content granular, apical cells narrowly rounded. Recorded in thermal springs in Europe (Belgium, France, Germany, Greece and Spain) and South Africa (Komárek & Anagnostidis, 2005).

***Phormidium lucidum* (Fig. 19).** Filaments somewhat curved, with mucilaginous sheaths and bright blue-green trichomes, slightly constricted at the finely granulated cross-walls (at both sides) and attenuated at the ends. Cells short; 3.5 long \times $5 \mu\text{m}$ wide. Recorded in thermal springs in Europe (Greece and Czech Republic) and

Table 2. Abundance and location of cyanobacterial species in Galician springs.

Cyanobacteria	Springs	Contribution (%)
* <i>Jaaginema angustissimum</i> (West & G.S. West) Anagnostidis & Komárek	Guitiriz	32.4
* <i>Leptolyngbya laminosa</i> (Gomont ex Gomont) Anagnostidis & Komárek	Outariz, Chavasqueira	20.5
<i>Symploca thermalis</i> Gomont	Burgas	10.3
<i>Leptolyngbya</i> sp ₁	Pantón	7.6
** <i>Aphanocapsa thermalis</i> Brügger	Cuntis	7.5
<i>Aphanocapsa conferta</i> (West & G.S. West) Komárková-Legnerová & Cronberg	Pantón, Guitiriz	7.3
<i>Leptolyngbya</i> sp ₂	Burgas, Cuntis	3.4
** <i>Chroococcus membraninus</i> (Meneghini) Nägeli	Guitiriz	1.9
** <i>Calothrix thermalis</i> Hasngirg ex Bornet & Flahault	Outariz, Cuntis	1.8
<i>Lyngbya martensiana</i> Meneghini ex Gomont	Pantón	1.8
** <i>Phormidium lucidum</i> Kützing ex Gomont	Outariz	1.2
* <i>Chroococcus thermalis</i> (Meneghini) Nägeli	Outariz	1.0
** <i>Leptolyngbya granulifera</i> (Copeland) Anagnostidis	Outariz	1.0
* <i>Phormidium boryanum</i> (Bory ex Gomont) Anagnostidis & Komárek	Burgas	0.8
** <i>Lyngbya thermalis</i> Anagnostidis	Burgas	0.4
* <i>Aphanothece bullosa</i> (Meneghini) Rabenhorst	Guitiriz	0.4
<i>Chroococcus minor</i> (Kützing) Nägeli	Pantón	0.4
** <i>Chroococciopsis thermalis</i> Geitler	Outariz	0.2
<i>Mastigocladus laminosus</i> Cohn ex Kirchner	Chavasqueira	0.2
<i>Gloeocapsa gelatinosa</i> Kützing	Burgas, Cuntis	0.1
** <i>Synechococcus bigranulatus</i> Skuja	Burgas	0.02
Total		100

Some of them are new records for Spain (**) and for Galician Region (*).

outside Europe (E Africa, Burma, India, USA) (Komárek & Anagnostidis, 2005).

***Symploca thermalis* (Fig. 20).** Long filaments entangled as a network. Trichomes pale to bright blue-green. Cells 1.2–1.6 × 2–2.4 µm long, sometimes nearly isodiametric. Cell content mostly homogeneous with one or two prominent granules on either side of the cross-walls. Apical cell rounded. Recorded in thermal waters (up to 52 °C) in Europe: Austria, Croatia, Czech Republic, France, Georgia, Greece, Hungary, Iceland, Italy, Portugal (Komárek & Anagnostidis, 2005) and Spain (Gonzalez Guerrero, 1944); outside Europe: Algeria, Azores, Cameroon, Canada, Israel, Russia, USA, etc. (Komárek & Anagnostidis, 2005).

Burgas was the spring that had the highest Shannon index (0.73 ± 0.27), and Chavasqueira the lowest (0.31 ± 0.48). Conversely, evenness was higher in Chavasqueira (0.88 ± 0.18) and lower in Outariz (0.44 ± 0.06) (Table 3). In general, a difference between hot and cold springs cannot be established with respect to these indexes. The maximum number of identified taxa (seven) was found in Burgas, and the minimum of only two different taxa was in Chavasqueira. Six species were recorded only once. The most species-rich genus is *Leptolyngbya* with four species. No species were found in common between cold and hot springs, not even in those of intermediate temperature considered.

Relationships between cyanobacterial communities and environmental variables

The nMDS ordination (Fig. 21) with an acceptable stress value of 0.26 showed a clear separation of the six springs based on the species found in them. In that ordination, it can be observed that the samples from Outariz, Burgas and Pantón were more uniform.

Based on the nMDS (Table 4), the most relevant variables were temperature ($R^2 = 0.72$, $P = 0.001$), conductivity ($R^2 = 0.57$, $P = 0.002$) and iron ($R^2 = 0.44$, $P = 0.002$). Temperature increases with the decrease of nMDS1 axis and with the increase of nMDS2 axis. This ordination allows to clearly separate the cold springs (right) from the hot springs (left), taking into account the cyanobacterial species present; Chavasqueira and Outariz standing in the middle of the ordination. Conductivity is an important variable on the nMDS1 axis, increasing to the left, where hot springs are located. The higher conductivity seems to be associated with the higher temperature springs. Therefore, the species found to the left of this axis are related to higher temperature and conductivity. The third variable that obtained significant weight on the nMDS1 axis was iron content. The iron content increases to the right of this axis, where the low temperature springs are located. The species located there could have greater need of this nutrient or lack mechanisms to capture it in conditions of less availability. The ANOSIM showed significant differences ($R = 0.98$; $P < 0.001$) in the cyanobacterial communities of the six springs. The SIMPER analysis

Table 3. Shannon's index and evenness of cyanobacteria found in six springs in Galicia.

Spring	Shannon's index	Evenness
Burgas	0.73 ± 0.27	0.65 ± 0.18
Chavasqueira	0.31 ± 0.48	0.88 ± 0.18
Outariz	0.67 ± 0.18	0.44 ± 0.06
Cuntis	0.47 ± 0.29	0.60 ± 0.12
Pantón	0.54 ± 0.13	0.70 ± 0.09
Guitiriz	0.44 ± 0.29	0.72 ± 0.19

Values are mean ± SD.

Table 4. Determination coefficients (R^2) and significance (P) of fitted vectors with ordination configuration on figure (***) 0.001, ** 0.01, * 0.05).

Variables	nMDS1	nMDS2	R^2	P
Temperature	-0.96871	0.2482	0.715	0.001***
pH	0.63679	0.77104	0.1325	0.242
Conductivity	-0.96906	-0.24681	0.5647	0.002**
Cl	-0.94085	-0.33881	0.2016	0.088
SO ₄	0.78800	-0.61567	0.0351	0.667
Ca	0.05643	-0.99841	0.2895	0.031*
Fe	0.88238	-0.47053	0.4388	0.002***
H ₂ S	-0.92052	0.39068	0.0037	0.951

also showed an overall average dissimilarity of 96.6%. Although no species stood out for contributing in a greater degree to this dissimilarity, the species that contributed most were *Leptolyngbya laminosa* and *Leptolyngbya* sp₁ (Table 5) with only 13.5 and 10.9%, respectively.

Based on SIMPER analysis, Table 6 shows the species that characterized each spring and its average contribution to similarity. It can be seen that each spring had its own species without coincidences and regardless of whether they correspond to cold or hot springs.

Discussion

As several reports indicate, one of the most important parameters for cyanobacteria species diversity in hot springs is temperature. Therefore, thermal gradients can determine the occurrence of the species in thermal springs. Results of this study support the fact that diversity of cyanobacteria species increases with increasing temperature, in contrast to Ferris et al. (1996a, 1996b) and Ward et al. (1998) who indicate that the diversity increased with decreasing temperature. However, it is necessary to consider that in those studies the temperature gradient studied was narrower than in this work and also, it was located in the range of high temperatures (around 50–75 °C). An increase in temperature in this range, towards extreme values, suggests a decrease in diversity. In this study with springs in Galicia, the range of temperatures covered was much wider, from

low to high temperatures, and an increase in diversity was observed. Maximum species diversity was observed in Burgas (≥ 40 °C), followed by Outariz (20–40 °C) and Pantón (≤ 20 °C). This result provides interesting information on the global effect of temperature on diversity.

Of the cyanobacterial species identified, the most diverse genus is *Leptolyngbya* with four species (*L. granulifera*, *L. laminosa*, *L. sp*₁, *L. sp*₂), followed by *Chroococcus* (*Ch. membraninus*, *Ch. minor*, *Ch. thermalis*) with three species and *Aphanocapsa* (*A. conferta*, *A. thermalis*), *Phormidium* (*Ph. boryanum*, *Ph. lucidum*) and *Lynghya* (*L. martensiana*, *L. thermalis*) with two species. Although the chemical environments in the springs were significantly different, none of the chemical variables measured was a significant determinant of the distribution.

Galician springs showed populations of cyanobacteria that varied in terms of composition and abundance. Of the 21 species of cyanobacteria identified, the number of taxa found was higher in warm waters (18 at four sites) than in cold springs (eight at two sites). This suggests that the diversity of cyanobacteria is higher in warm than in cold environments. This could be due to some physical and chemical features such as the high amount of iron in cold sources: 302.5 $\mu\text{g L}^{-1}$ in Guitiriz and 73.5 $\mu\text{g L}^{-1}$ in Pantón as well as the high pH value especially in Guitiriz (9.5) which would greatly hinder the development of cyanobacteria species causing a decrease in biodiversity in these cold sources. Iron is essential for growth but is highly toxic if intracellular iron levels are not controlled, given its tendency to generate oxidative stress (Imlay, 2003). It is known that the presence of 1 mM of FeSO₄ in the culture medium is able to inhibit the *in vitro* growth of *Anabaena* sp. (Lammers & Sanders-Loehr, 1982). In this way, although it is important for the cyanobacteria to develop mechanisms to ensure the supply of iron for its vital functions, it will be equally important to ensure the intracellular iron content is kept below toxic levels, so all iron incorporation, transport and storage machinery is strictly regulated based on iron availability and use by the cell.

Within warm waters, there are common taxa such as *Gloeocapsa gelatinosa* and *Leptolyngbya laminosa* that were found both at high and intermediate temperatures, demonstrating their thermal amplitude, unlike what occurs at the Pancharevo hot spring in Sofia, Bulgaria (Lukavský et al., 2011). The most abundant species was *Jaaginema angustissimum* in Guitiriz, coinciding with the thermal springs in the province of Horzogan, Iran (Arman et al., 2014), followed by *Leptolyngbya laminosa* in Outariz and Chavasqueira, as well as in the

Table 5. Results of the SIMPER analyses for the dissimilarity of the springs based in the cyanobacterial species found in them.

Taxon	Av. dissim	Contrib. %	Cumulative %
<i>Leptolyngbya laminosa</i>	13.07	13.53	13.53
<i>Leptolyngbya</i> sp. ₁	10.56	10.94	24.47
<i>Aphanocapsa thermalis</i>	10.39	10.75	35.23
<i>Jaaginema angustissimum</i>	9.771	10.12	45.34
<i>Symploca thermalis</i>	9.514	9.851	55.19
<i>Mastigocladus laminosus</i>	8.578	8.882	64.07
<i>Leptolyngbya</i> sp. ₂	7.165	7.418	71.49
<i>Aphanocapsa conferta</i>	4.687	4.853	76.35
<i>Lyngbya martensiana</i>	3.934	4.074	80.42
<i>Calothrix thermalis</i>	3.6	3.728	84.15
<i>Phormidium lucidum</i>	2.923	3.026	87.17
<i>Chroococcus thermalis</i>	2.208	2.286	89.46
<i>Leptolyngbya granulifera</i>	2.117	2.192	91.65
<i>Phormidium boryanum</i>	1.673	1.733	93.38
<i>Chroococcus minor</i>	1.663	1.722	95.11
<i>Chroococcus membraninus</i>	1.232	1.276	96.38
<i>Gloeocapsa gelatinosa</i>	1.106	1.145	97.53
<i>Lyngbya thermalis</i>	1.025	1.061	98.59
<i>Aphanothece</i> sp.	0.5409	0.5601	99.15
<i>Chroococciopsis thermalis</i>	0.4697	0.4863	99.63
<i>Synechococcus bigranulatus</i>	0.2199	0.2277	99.86
<i>Aphanothece bullosa</i>	0.1331	0.1378	100

Table 6. Contributions (based on SIMPER analysis) of the main species that characterized the springs.

Spring	Species	Average contribution to the similarity
Burgas	<i>Symploca thermalis</i>	49.84
	<i>Leptolyngbya</i> sp. ₂	23.78
Chavasqueira	<i>Mastigocladus laminosus</i>	36.49
Outariz	<i>Leptolyngbya laminosa</i>	53.14
	<i>Chroococcus thermalis</i>	11.25
Cuntis	<i>Aphanocapsa thermalis</i>	56.26
Pantón	<i>Leptolyngbya</i> sp. ₁	61.95
Guitiriz	<i>Jaaginema angustissimum</i>	50.61

thermal springs in Algeria (Amarouche-Yala *et al.*, 2014); and *Symploca thermalis* in Burgas as in the Pancharevo hot spring in Sofia, Bulgaria (Lukavský *et al.*, 2011) and in hot springs in northern Thailand (Sompong *et al.*, 2005). In fact, this last species, considered thermophilic, is one of the species that characterized the Burgas samples (Table 6 and Fig. 21) due to its abundance in this hot spring. Something similar occurred with *Mastigocladus laminosus*, another species considered thermophilic (Miller, 2007), which was abundant in the Chavasqueira samples, another of the hot springs sampled.

In the hot springs, the coccoid species never exceed the filamentous ones, whereas in the cold springs, the filamentous species never exceed the coccoid. In Burgas ($\geq 40^\circ\text{C}$), the spring with the greatest species diversity, there are twice as many filamentous species as there are

coccoid ones. The presence of *Synechococcus bigranulatus* coincides with Skirmisdottir *et al.* (2000) who indicated that *Synechococcus* are typical of thermal gradients from $50\text{--}75^\circ\text{C}$. However, Norris *et al.* (2002) stated that *Synechococcus* could also develop at lower temperature values. In Outariz ($20\text{--}40^\circ\text{C}$), the filamentous species predominate over the coccoid, coinciding with Ward and Castenholz (2002) and Sompong *et al.* (2005) for the lower level of thermophilia. In Guitiriz ($\leq 20^\circ\text{C}$), the coccoid species predominate over the filamentous ones.

In general, there are significant differences in the cyanobacterial communities from the six springs studied; in fact, we have not found any species that appears in all the temperature gradients studied, unlike Arman *et al.* (2014), who did detect the genera *Jaaginema* and *Synechococcus* in all analysed hot springs from Hormozgan, Iran. These results seem to indicate that it is difficult to establish a characteristic cyanobacterial flora for the waters of the Galician springs as occurs with the diatom flora (Leira *et al.*, 2017). In fact, none of the taxa was common to the six springs, and only five species (*Aphanocapsa conferta*, *Calothrix thermalis*, *Gloeocapsa gelatinosa*, *Leptolyngbya laminosa* and *L. sp.*₂) were found in two springs. Furthermore, 77.3% of the taxa were only found in a single spring. It should be added that some taxa in these Galician springs were widely distributed species (probably habitat generalists) with wide ranges of tolerance to the main environmental variables.

All the hot springs studied are accessible to the public and are popular tourist destinations. Therefore, human activity may have caused disturbance or disruption to the cyanobacterial mats. The water outlet is still semi-natural, exposed to light and a suitable place for the growth of thermophilic cyanobacteria. It is important to protect this important ecological habitat, according to Lukavský et al. (2011), and to conserve this refugium of interesting and valuable genotypes, since eight new species were recorded for Spain (*Aphanocapsa thermalis*, *Calothrix thermalis*, *Chroococcus membraninus*, *Chroococciopsis thermalis*, *Leptolyngbya granulifera*, *Lyngbya thermalis*, *Phormidium lucidum*, *Synechococcus bigranulatus*) and five for the Galician region (*Aphanothece bullosa*, *Chroococcus thermalis*, *Jaaginema angustissimum*, *Leptolyngbya laminosa*, *Phormidium boryanum*). It is essential to establish some protective measures to prevent the deterioration of the springs, considered as endangered habitats, according to Cantonati et al. (2009).

The importance of expanding our knowledge on thermophilic microorganisms stems from the development of novel secondary metabolites for our health, food and environment. Cyanobacteria represent a group of diverse microorganisms that inhabit a wide range of environments, even in extreme conditions. Studies of thermophilic microorganisms have shown considerable biotechnological potential due to their optimum growth and metabolisms at high temperatures ($\geq 50^\circ\text{C}$), which is supported by their thermostable enzymes. Cyanobacterial communities present in high-temperature ecosystems account for a large part of the total ecosystem biomass, and productivity can be exploited to generate several value-added products of agricultural, pharmaceutical, nutraceutical and industrial relevance. Thermophilic cyanobacteria have been extensively studied for biotechnological applications (Patel et al., 2019). These include the production of pigments (carotenoids and phycobiliproteins), nutrition supplements (omega-3 and omega-6 polyunsaturated fatty acids), renewable fuels (bioethanol, biodiesel and hydrogen), fertilizers and secondary metabolites including exopolysaccharides, vitamins, toxins and biologically active compounds with antiviral, antiseptic, antifungal and anticancer activities. Moreover, these microorganisms are also suitable for bioremediation of agroindustrial wastewater and biodegradation of oily wastewater.

Conclusions

It is difficult to establish a characteristic cyanobacterial flora for the thermal waters of the Galician springs since there are significant differences in the communities from

the six sites studied. Galician springs showed populations of cyanobacteria that varied in terms of composition and abundance. Temperature is the environmental parameter that most influences the distribution of cyanobacteria in springs of Galicia. So, the diversity of cyanobacteria in these environments increases with increasing temperature. Iron content was the next most influential variable, in this case with a negative correlation. Of the 21 species identified, the most diverse genus is *Leptolyngbya* with four species, followed by *Chroococcus* with three species and *Aphanocapsa*, *Phormidium* and *Lyngbya* with two species. The most abundant species was *Jaaginema angustissimum*, followed by *Leptolyngbya laminosa* and *Symploca thermalis*. Moreover, of the total species of cyanobacteria, eight have been cited for the first time in Spain and five are new in the Galician region, indicating the need to protect these environments due to their interest for algal diversity. Therefore, we suggest that, during future studies on springs in Spain, an interesting aspect that should be considered is the culture approach in order to keep the cyanobacterial diversity of the springs in culture collections.

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Authors contributions

M.C.L., A.D.A. and R.M. conceived and designed the sampling. M.C.L., A.D.A. and E.T. analysed the data. M.C.L., A.D.A. and E.T. wrote the paper and contributed to the general discussion, revision and manuscript editing. All authors read and approved the final manuscript.

Disclosure statement

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