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Article

Frequent Microalgae in the Fountains of the Alhambra and Generalife: Identification and Creation of a Culture Collection

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Abstract: Cyanobacteria, green algae and diatoms are significant factors in the biodeterioration of stone cultural heritage sites, and specifically fountain monuments, due to the constant presence of water. In this study, samples were taken from different fountains in the Alhambra and Generalife, which are among the Spanish monuments of greatest historical and artistic value and which together were declared a World Heritage Site by UNESCO in 1984. The aim was to identify which species of colonising microalgae are most frequent and to obtain monoalgal cultures from them. From a conservation point of view, it is interesting to identify which algae are growing in these fountains and how they behave in order to develop new methods to control their growth. The most abundant groups of algae in our samples were green algae and cyanobacteria. The most common genera in the former group were *Bracteacoccus*, *Chlorosarcina*, *Chlorosarcinopsis*, *Apatococcus* and *Klebsormidium*. As for cyanobacteria, the most abundant genera were *Phormidium*, *Calothrix*, *Leptolyngbya*, *Chamaesiphon*, *Pleurocapsa* and *Chlorogloea*. Using our collected samples, 10 genera of green algae and 13 genera of cyanobacteria were isolated, thereby constituting the base samples for the creation of a reference collection of living algae from the Alhambra and Generalife contexts, which can be used in subsequent studies to develop new types of treatment against biodeterioration.

Keywords: green microalgae; cyanobacteria; diatoms; biodeterioration; Alhambra; Generalife; conservation; information modelling; stone fountains; cultural heritage

1. Introduction

Biodeterioration is defined as "any undesirable change in the properties of a material caused by the vital activities of organisms" [1]. Microorganisms such as bacteria, fungi and algae are some of the agents that affect cultural heritage. These include microalgae (microscopic algae), which are photoautotrophic organisms capable of growing in media not abundant in nutrients, such as stone, and which proliferate in places with water or high relative humidity levels. In particular, they play an especially relevant role in fountains and ornamental pools, because of the constant presence of water. These organisms are the main agents involved in the biodeterioration of such cultural assets, causing a range of aesthetic, physical and chemical damage, which has been studied over time around the world [2–8].

1.1. Biodeterioration Caused by Microalgae

Algae are a polyphyletic group of organisms, i.e., not all of them come from the same evolutionary ancestor. Within this group we can find enormous diversity in all aspects. For decades, cyanobacteria were called blue-green algae and were grouped with other algae, mainly because of their ability to photosynthesise. Today, however, cyanobacteria are no longer considered to be algae, but rather are considered to be prokaryotic organisms belonging to the domain of bacteria, whereas all algae are eukaryotic organisms. Nevertheless, in this work we have included cyanobacteria in the term "microalgae", since both cyanobacteria and eukaryotic microalgae share important similitudes from an ecological point of view—they both carry out oxygenic photosynthesis, are pioneers in stone colonisation and cause biodeterioration through similar processes.

The microalgae that affect the state of conservation of fountains and other stone monuments can be epilithic (growing on the surface of the stone) or endolithic (growing inside it) [9], with the latter being the most damaging and difficult to remove. In any case, the microalgae grow and produce biofilms, which are composed of a set of organisms and the substances they excrete. The microalgae are often embedded in the matrix formed by extracellular polymeric substances (EPS) secreted by the cells themselves. These biofilms affect the stone in different ways.

Firstly, they produce an aesthetic alteration, since they form a patina with different natural colourings (light or dark green, orange, brown, etc.) on the stone. Furthermore, biofilms can retain atmospheric dust, pollutants from the air and substances dissolved in water that give them a darker colour. This phenomenon is known as "soiling" [10].

These biofilms also cause physical deterioration. Many species of algae and cyanobacteria have sheaths that produce mucilage, which allows them to adhere to the substrate. The mucilage is also used for gliding [11] and to retain water, so that they can survive drought conditions. On absorbing water, the sheaths' volumes vary considerably and their continual expansion and contraction causes the gradual destruction of stony material, which progressively loses granules that go on to form part of the biofilm [12]. The water retention is a fundamental aspect—it fosters mechanical damage due to frost weathering with sharp temperature changes and also allows for the growth of other algae with greater water requirements, so that the communities become more complex. It may also help to support other, more damaging organisms such as fungi and bryophytes [10]. If there is an excessive proliferation of algae, they can affect how the fountains work, for example by blocking spouts, which can speed up other kinds of deterioration.

Lastly, microalgae also cause chemical damage as a result of their metabolism. They directly damage the stone by producing organic acids (pyruvic, glycolic, lactic, acetic, succinic or alpha-ketoglutaric) as a result of respiration. They can also give rise to biotransference phenomena by producing polysaccharides capable of reacting with metals and leading to salts [10]. However, the main damage caused by microalgae is the formation of carbonate crusts on stone as a result of photosynthetic metabolism. Due to the withdrawal of carbon dioxide from the medium and to other biochemical processes, the pH increases and fosters the precipitation and aggregation of calcium carbonate, forming layers of mineral crusts that can end up covering the original materials. All of the taxonomical groups of microalgae are capable of producing this phenomenon, but it is more apparent in green algae and cyanobacteria [13]. In addition to causing aesthetic damage, these crusts foster physical biodeterioration due to thermal contraction and expansion to a different extent than the substrate, leading to stresses that weaken it, and also due to frost weathering, since they have a porous structure that increases water retention further [14]. They even end up burying the algae that have generated them, which then become endolithic and protected from environmental adversity and treatments, such as cleaning or biocides, thereby making it much more difficult to remove them.

1.2. Microalgae in the Alhambra

The Alhambra and the Generalife, together named a UNESCO World Heritage site in 1984, form one of the monument complexes of greatest historical and artistic value in Spain, meaning their conservation is of great importance. Given the importance of water in Nasrid and Islamic art in general, "*alberca*" reservoirs abound throughout the site, together with channels and fountains (including iconic ones such the Fountain of the Lions), which are affected by colonisation from microalgae (Figure 1a,b). Although the fountains undergo continual maintenance, these microorganisms are not completely eliminated. Despite the continual investment in chemical and mechanical means of control, the natural process of colonisation starts over.

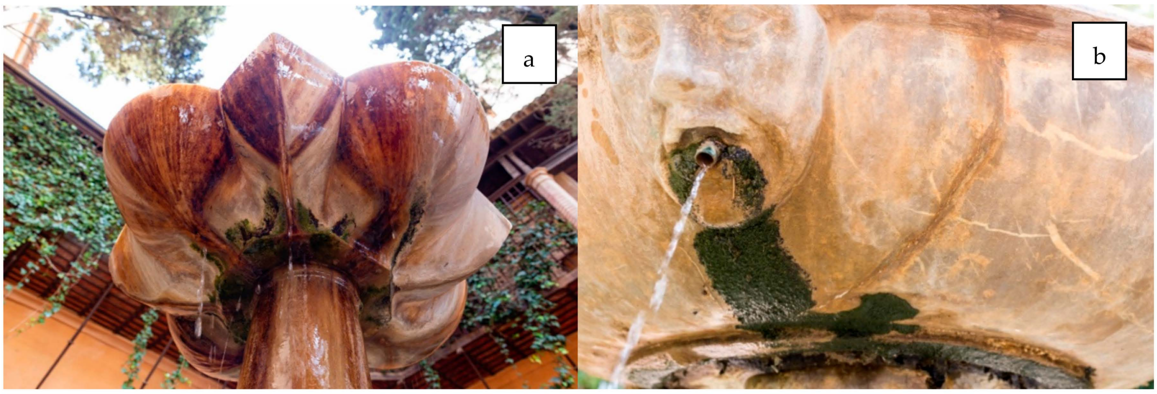

Figure 1. (**a**) Two different phycofilms on the *Fuente del Patio de la Reja* at the Nasrid Palaces. (**b**) Algal mat growing on the *Fuente del Patio de la Sultana* at Generalife. Photos by G. Alfano.

From a conservation point of view, an important step will be to learn the structure and functioning of the biofilms—which species they are composed of, which species are the most resistant or harmful and which conditions help them develop—in order to know how to create new methods in order to halt the proliferation of the microorganisms. There are a variety of previous studies on the biodeterioration of fountains in the Alhambra, many of which focus on biodeterioration due to microalgae, their classification and possible treatments [13–18]. Our work was intended to update the knowledge on the types of microalgae growing in different fountains throughout the Alhambra and Generalife today, establishing which genera are the most common and beginning to create a culture collection of algae with species from these sites.

1.3. Objectives

The main objectives of this study were as follows:

- To identify the most common types of microalgae in the fountains of the Alhambra and Generalife today.
- To make unialgal cultures of the species found in the Alhambra in order to begin creating a culture collection of living microalgae available for subsequent studies on new kinds of treatment to keep biodeterioration under control.
- To design a data model and develop a database for storing and retrieving sample details and analytical values from the collection.

2. Materials and Methods

2.1. Sampling

In total, 21 fountains in the Alhambra and Generalife were studied, with 120 microalgae samples being taken (Table 1, Figures 2 and 3). The greatest number of samples was taken from the monumental fountains of the Nasrid Palaces (60%) due to their enormous cultural interest, although fountains from other areas were also selected in order to make a general assessment of the most common microalgae species colonising the monument complex.

Table 1. Fountains studied in different areas of the Alhambra and numbers of samples collected. In the last two columns, in brackets, the numbers of samples taken corresponding to different materials and environments are shown.

Zone	Fountain	No. of Samples	Material	Environment
Nasrid Palaces	1. Fuente del Patio del Cuarto Dorado (Mexuar fountain)	7	marble (5), marble and metal (1), mortar (1)	submerged (6), amphibious (1)
	2. Fuente-guitarra N del Patio de los Arrayanes (North "guitar" fountain of the Court of the Myrtles)	15	marble (10), metal (5)	submerged (9), amphibious (5), aerial (1)
	3. Fuente-guitarra S del Patio de los Arrayanes (South "guitar" fountain of the Court of the Myrtles)	6	marble (5), metal (1)	submerged (4), amphibious (2)
	4. East channel of the Court of the Myrtles	2	marble (2)	submerged (1), amphibious (1)
	5. Fuente de los Leones (Fountain of the Lions)	21	marble (15), mortar (3), metal (3)	submerged (4), amphibious (13), aerial (4)
	6. Fuente del Patio de la Reja (Fountain of the Court of the Grille)	11	marble (11)	submerged (8), amphibious (3)
	7. Fuente del Patio de Lindaraja (Fountain of the Court of the Lindaraja)	10	marble (10)	submerged (1), amphibious (5), aerial (4)
Partal	8. Fuente-guitarra junto a la Torre de las Damas	6	marble (5), metal (1)	submerged (4), amphibious (2)
	9. Pilar (Basin) debajo del Mirador del Partal	5	brick (5)	submerged (1), amphibious (2), aerial (2)

Table 1. *Cont.*

Zone	Fountain	No. of Samples	Material	Environment
Casa del Arquitecto	10. Inner fountain of the court with oranges	1	painted plaster (1)	aerial (1)
Alcazaba	11. Pilar E. del Jardín de los Adarves (East basin of the Garden of the Ramparts)	3	marble (3)	amphibious (2), aerial (1)
Secano	12. Fuente de la Glorieta del Secano	5	marble (5)	submerged (3), amphibious (2)
Generalife	13. Fuente S de los Jardines Bajos (South fountain of the Lower Gardens)	2	marble (2)	submerged (1), amphibious (1)
	14. Fuente central del Patio de la Sultana (Central fountain of the Sultana's Court)	7	marble (6), brick (1)	submerged (2), amphibious (2), aerial (3)
	15. Pretil de la Escalera del Agua (The handrail of the Water Stairway)	4	glazed tile (2), plaster (2)	submerged (2), amphibious (2)
Walkways	16. Fuente del Tomate (Tomato fountain)	3	marble (3)	submerged (3)
	17. Fuente de Ángel Ganivet	3	bronze (1), mortar (2)	submerged (2), amphibious (1)
	18. Pilar de Carlos V	3	marble (3)	submerged (1), amphibious (2)
	19. Pilar de Washington Irving	3	marble (3)	amphibious (2), aerial (1)
	20. Pilar de la Puerta de las Granadas (Basin of the Gate of the Pomegranates)	2	marble (2)	aerial (2)
Carmen de Bellavista	21. Fuente derecha de la 2ª terraza (Right fountain of the 2nd terrace)	1	marble (1)	amphibious (1)
Total samples		120		

Figure 2. Sampling from the Fountain of the Lions. Photo by G. Alfano.

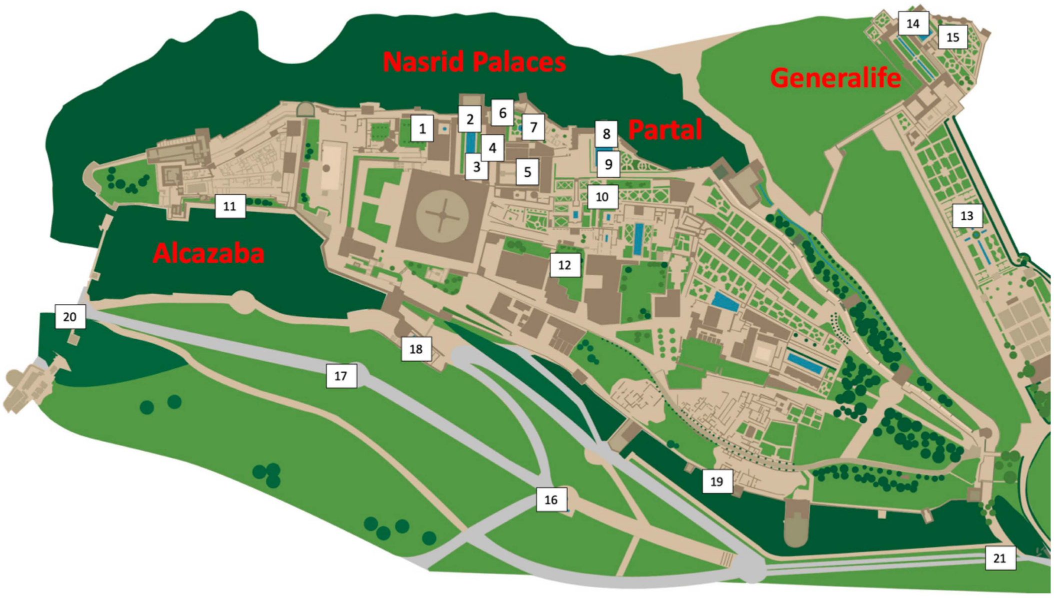

Figure 3. Sampling points in the monumental complex of the Alhambra and the Generalife. The labels correspond to Table 1.

The main materials used in the construction of the fountains studied are white marble from Macael (Almería, Spain) and limestone from Sierra Elvira (Granada, Spain). Samples were also taken from the surface of the metal spouts in several fountains, and occasionally from mortar, brick, plaster and glazed tile.

Seasonal samples were taken in spring, summer, autumn and winter throughout 2017 and 2018. The sampling points chosen were the surfaces of fountains, where generally abundant macroscopic growth of epilithic microalgae had been observed (Figures 4a and 5a). For each of them, the appearance of the microalgal communities was taken into account, as well as the environment they belong to, according to the water available—submerged, amphibious or aerial. The submerged zones are those below the water level, as well as those exposed to continually running water, for example inside the spouts or the outer surfaces of basins where the water overflows. The amphibious zones are those with water available intermittently, such as surfaces that are splashed by the spouts. Lastly, we considered aerial zones to be those that do not receive water directly, such as the inner surfaces of basins above the waterline. As for the appearance of the communities, we can distinguish: patinas (sheens) when they are of scant thickness and less structural complexity; mats of greater thickness and complexity with filamentous species; and mineral crusts, which may arise due to any of the aforementioned formations. On taking the different samples, a variety of fountains and environments were chosen to take into account the greatest possible variation. In some cases, samples were also taken in the same zone at different times of year to check if there were variations in the types of species. All of the sampling processes were photographically documented (i.e., fountain, sampling point, sample, etc.), giving a total of around 450 high quality images.

Figure 4. (**a**) Sampling a mineralising crust with a scalpel at *Fuente N del Patio de los Arrayanes*. (**b**) Two sheeted crusts from perpendicular angles with buried communities. Photos by G. Alfano.

Figure 5. (**a**) South fountain of the Lower Gardens. The base of the basin, where a dark green mat can be seen, was one of the sampling points; (**b**) Taking a sample of mineral crust in the *Escalera del Agua* (Water Stairway); (**c**) Sample of mineral crust taken in the Washington Irving basin. Photos by G. Alfano.

2.2. Procedures

The samples were taken with the help of tweezers and a scalpel, taking the utmost care not to damage the original materials (Figures 4a and 5b). The fresh samples were stored in a polyethylene sample collection vial with water from the same fountains from which they were taken (Figure 5c). In the laboratory, they were refrigerated until their observation. After observation and creation of the cultures, the samples were fixed in a glutaraldehyde and 25% glycerol solution (Sigma Aldrich, Madrid, Spain) to be permanently conserved for subsequent study.

The microalgae present in each sample were identified in the phycology lab at the University of Granada, using a binocular Olympus SZX10 microscope and an inverted optical Nikon Eclipse TS100 microscope. The following references were used for the taxonomical identification [19–23]. In each sample, the different microalgal species were identified according to their proportion by assigning an index (+–5) representing the following percentages of the total: + = 0–1%; 1 = 1–10%; 2 = 10–25%; 3 = 25–50%; 4 = 50–75%; 5 = 75–100%.

Microalgal cultures were made in a solid medium in agar plates at 2%. A high concentration of agar (Sigma-Aldrich, Madrid, Spain) was used because we intended to cultivate benthonic species that grow on stone. The media for cultivation used were Bold's Basal Medium (BBM) (Phyto-Tech, Lenexa, KS, USA) for green algae and BG11 (Sigma-Aldrich, Madrid, Spain) for the cyanobacteria. Diatoms were not cultivated since they have stricter nutritional requirements and are the most uncommon group of the three.

The streaking method was used to isolate microalgae on plates using fresh samples, successive isolations and re-streaking until unialgal cultures were obtained. The plates were incubated at 22 °C with a cycle of 12 h of light/darkness using fluorescent lamps that provided a light intensity of 800 lux. On average, the cultures took about two weeks to develop, but the time varied depending on multiple factors, so the re-streaking was carried out depending on the needs in each case.

2.3. The Database

From the planning phase of this study, it was clear that the information collected would have to be processed digitally in order to provide the required features of data consistency, automated backups, multimedia, versioning, automatic quantifications and simultaneous user access. For this reason, two of the fundamental steps towards the creation of the algae culture collection were the design of a specific data model (tables, attributes, constraints and relationships) and creation of a multimedia, geographically enabled database to store and retrieve data collected during the sampling process and in the lab.

The main tables of the database are used to register the collected samples, the monuments (fountains, pools, etc.) and the identified microalgae; the sample represent the central table, which is related to both the monuments (one-to-many cardinality) and microalgae (many-to-many cardinality). Several attributes were added in order to register a wide variety of values, such as the date, location, environment, media and description; classes, genera, species, colour, communities, biotype and biotope; applied treatments, cultivation procedures and microphotographs; material and chronology.

The database is simultaneously accessible by several users with individual accounts and different roles. Stored data can be retrieved using a unified search box or through a multifaceted search engine, supporting filters and Boolean operators. Every change in each record is registered in a different version, so individual states can be restored or compared between each other. The database's architecture is modular so that additional workflows can be introduced in the future, for example to continue tracing the evolution of biodeterioration through time and space.

3. Results and Discussion

3.1. Microalgae Identification

Firstly, it should be noted that the fountains in the Alhambra and Generalife undergo continual maintenance. In addition to general cleaning, all of the studied fountains are treated with algicide. Chlorine tablets are also added to many of them. In the specific case of the Fountain of the Lions, the water flowing through gets special treatment, since the circulating water is chlorinated and purified independently from the rest of the fountains of the monumental complex. This means that the algae colonising these fountains exist in conditions that are far from natural and that they are generally resistant to such treatment, since it does not manage to eliminate them completely.

Fundamentally, as seen in previous studies [15,16], the microalgae colonising the selected fountains largely belong to the large groups of green algae (Chlorophyta and Charophyta), cyanobacteria (Cyanobacteria) and diatoms (Bacillariophyta). Once in a while, some Euglenophyceae have been found in the "guitar" fountain of the Ladies Tower (Torre de la Damas, Granada, Spain), and a Phaeophyceae (*Pleurocladia* sp.) sample was found in the southern "guitar" fountain of the Court of the Myrtles (Patio de los Arrayanes), but those species were only seen in spring in those two fountains. Table 2 shows a list of the fountains studied and the most common genera of microalgae in each.

Table 2. The most common genera of microalgae in each of the fountains studied. For each fountain, the set of samples taken was considered, along with the different sampling points, and where applicable the different times of year. The genera that appear most often and in greater abundance were chosen. For each genus, the total number of samples in which it is present (first number) and the number of samples in which its index is 3 or higher (second number; see 2.2. Procedures) are shown in brackets.

Fountain		Most Common Genera		
		Green Algae	**Cyanobacteria**	**Diatoms**
1.	Mexuar fountain	*Bracteacoccus* (3-2), *Chlorosarcinopsis* (2-1)		*Navicula* (3-1), *Nitzschia* (2-2)
2.	North "guitar" fountain of the Court of the Myrtles (*Patio de los Arrayanes*)	*Chlorosarcinopsis* (5-2)	*Dichothrix* (5-3), *Phormidium* (6-5), *Leptolyngbya* (9-6),	
3.	South "guitar" fountain of the Court of the Myrtles		*Phormidium* (4-2), *Calothrix* (4-1), *Chlorogloea* (1-1), *Pleurocapsa* (3-1)	
4.	East channel of the Court of the Myrtles		*Leptolyngbya* (2-2), *Pleurocapsa* (2-1)	
5.	Fountain of the Lions	*Klebsormidium* (3-2)	*Phormidium* (7-6), *Leptolyngbya* (9-2), *Cyanosarcina* (5-4)	

Table 2. *Cont.*

	Fountain	Most Common Genera		
		Green Algae	**Cyanobacteria**	**Diatoms**
6.	*Patio de la Reja* fountain	*Chlorosarcina* (7-5)	*Symploca* (1-1), *Leptolyngbya* (5-4), *Chamaesiphon* (3-2), *Calothrix* (2-1)	
7.	*Patio de Lindaraja* fountain	*Chlorococcum* (4-2), *Chlorosarcinopsis* (4-1), *Klebsormidium* (5-2)	*Chamaesiphon* (6-3), *Phormidium* (2-2)	
8.	Ladies Tower (*Torre de las Damas*)	*Pleurastrum* (2-2)	*Symploca* (3-2), *Phormidium* (3-2), *Leptolyngbya* (2-1)	
9.	*Mirador del Partal* basin	*Bracteacoccus* (4-4)		*Navicula* (2-1)
10.	Inner fountain of the court with oranges	*Klebsormidium* (1-1)		
11.	*Jardín de los Adarves* east basin	*Bracteacoccus* (3-2), *Choricystis* (1-1)		
12.	*Glorieta del Secano* fountain	*Bracteacoccus* (4-3)	*Chamaesiphon* (2-1), *Cyanosarcina* (1-1)	
13.	*Jardines Bajos* South fountain		*Phormidium* (2-2)	
14.	*Patio de la sultana* central fountain	*Gongrosira* (3-1), *Bracteacoccus* (3-3)	*Phormidium* (3-1), *Lyngbya* (1-1)	*Navicula* (4-1), *Cymbella* (3-1)
15.	*Escalera del Agua* handrail	*Pseudopleurococcus* (1-1), *Leptosira* (2-1), *Chlorosarcina* (2-2)	*Myxosarcina* (1-1), *Chlorogloea* (1-1)	
16.	*Fuente del tomate*	*Bracteacoccus* (2-1)	*Chlorogloea* (1-1), *Chamaesiphon* (1-1), *Phormidium* (2-1)	
17.	Ángel Ganivet pool fountain	*Bracteacoccus* (2-2)	*Leptolyngbya* (1-1)	
18.	Carlos V basin	*Bracteacoccus* (3-1)	*Chamaesiphon* (2-1)	
19.	Washington Irving basin	*Bracteacoccus* (2-1)		

Table 2. *Cont.*

Fountain		Most Common Genera		
		Green Algae	Cyanobacteria	Diatoms
20.	*Puerta de las Granadas* basin	*Bracteacoccus* (1-1)	*Chroococcopsis* (1-1)	
21.	Right fountain of the 2nd terrace of the *Carmen de Bellavista*		*Calothrix* (1-1), *Phormidium* (1-1)	

In general, diatoms are the least common type of microalgae of the three groups studied. The most common genera are *Navicula*, *Nitzschia*, *Cymbella* and *Achnanthes*, all of which are unicellular; in other words, the cells appear individually and do not form aggregates adhering strongly to surfaces. Of these four genera, *Navicula*, *Nitzschia* and *Cymbella* were the ones that generally appeared most often. All of these diatoms appear to a greater or lesser extent as part of more complex communities of cyanobacteria or green algae, except one specific case in which a monospecific community of *Achnanthes* sp. was found on the surface of the basin at the Fountain of the Lions in a submerged environment.

Among the green algae, the unicellular coccoid algae were very widespread in many fountains. The most common genera were *Chlorococcum*, *Bracteacoccus*, *Chlorosarcina* and *Chlorosarcinopsis* (Figure 6a). The latter two are able to form generally compact cellular aggregates; less frequent (but still common) were the *Chlorella* and *Choricystis* genera.

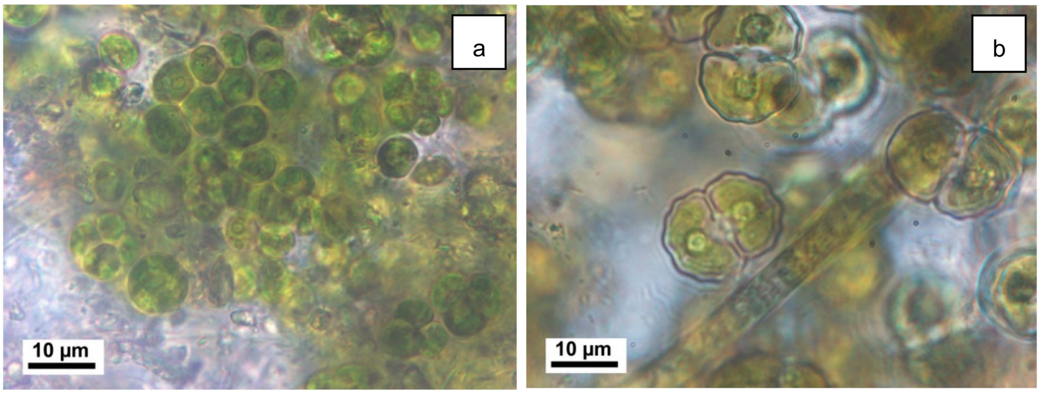

Figure 6. Photographs of samples taken using optical microscopy: (**a**) *Chlorosarcinopsis* sp. cells can be seen on the left; (**b**) *Cosmarium* sp. cells can be seen on the right, along with a filament of cyanobacteria.

In addition to the coccoid algae, we also found pseudoparenchymatic green algae—*Pleurastrum* sp. appears on the "guitar" of the Ladies Tower and *Gongrosira* sp. appears in the pool of the central fountain of the Court of the Sultana, both in submerged zones; while *Leptosira* sp. and *Pseudopleurococcus* sp. grow in the Water Stairway's handrail in amphibious and submerged zones. As for filamentous algae, the *Klebsormidium* genus is common, which is also capable of growing in aerial zones. The filamentous alga *Cladophora* sp. has also been found sporadically (in the Lindaraja fountain and the *mirador del Partal* basin), as has *Stigeoclonium* sp. (*Patio de los Arrayanes* southern "guitar" fountain). *Stichococcus* sp. appears in amphibious and aerial zones of the Fountain of the Lions and in the handrail of the *Escalera del Agua*, although not abundantly. This alga is normally unicellular, although it can form lax filaments that disaggregate easily. Other green algae genera that are very widespread, although their proportions in the samples are usually small, were *Scenedesmus* sp., which is characteristic for the formation of coenocytes; *Cosmarium* sp. (unicellular; Figure 6b); and *Apatococcus* sp. (pseudoparenchymatic).

As for the cyanobacteria, we can distinguish between the Chlorococcales order, whose cells group together in colonies (and are occasionally filamentous in appearance), and truly filamentous algae. The most common chlorococcal cyanobacteria among all the fountains studied were *Pleurocapsa* sp. (Figure 7a), *Chamaesiphon* sp. (Figure 7b) and *Chlorogloea* sp., followed by *Chroococcidiopsis* sp., which is less common or abundant. *Cyanosarcina* sp. is very common in amphibious zones in the Fountain of Lions and the fountain of the *Glorieta del Secano*, while *Myxosarcina* sp. grows in crusts on the southern "guitar" of the *Patio de los Arrayanes* and the *Escalera del Agua*. On the Fountain of the Lions and the basin of the *Puerta de las Granadas* there were species of the genus *Chroococcopsis*. Among the filamentous cyanobacteria, the most common genera were *Phormidium* (Figure 8a,b), *Calothrix*, *Leptolyngbya*, *Lyngbya* and *Schizothrix*. The former three appear in a large number of fountains and are relatively abundant. *Symploca* sp. appears abundantly in submerged zones of the "guitar" of the *Torre de las Damas* and in the fountain of the *Patio de la Reja*. In the spout of the northern fountain in the *Patio de los Arrayanes*, where samples have been taken in different seasons, a dark mat forms in which there is a great presence of *Dichothrix* sp.

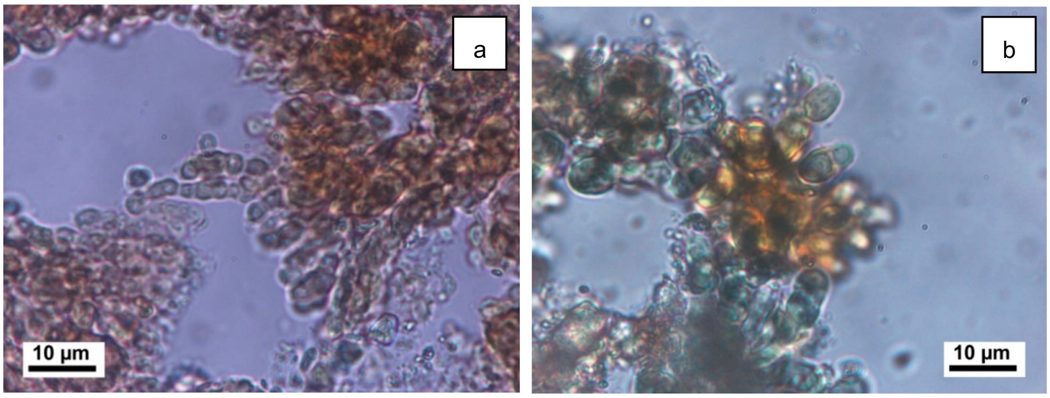

Figure 7. Photograph of samples taken using optical microscopy: (**a**) *Pleurocapsa* sp. cells are shown on the left; (**b**) *Chamaesiphon* sp. cells are shown on the right.

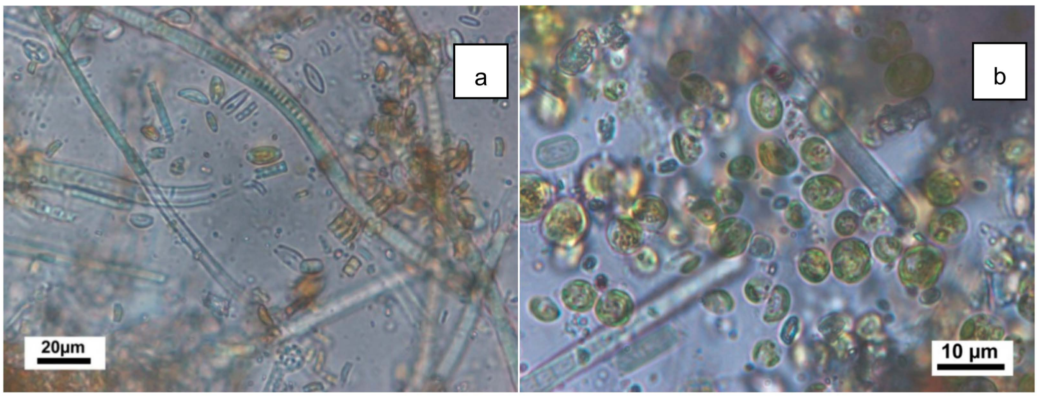

Figure 8. Photograph of samples taken using optical microscopy: (**a**) diatoms of the *Cymbella, Navicula and Achnanthes* genera and filaments of *Phormidium* sp. can be seen on the left; (**b**) on the right, there are cells of *Bracteacoccus* sp. and filaments of *Phormidium* sp.

Normally, in each sample we can find generally complex communities made up of two or more different species of microalgae. However, occasionally the sheens (structures of less complexity) are composed of a single species. In the vast majority of cases, these species belong to the unicellular coccoid green algae group, such as *Chlorosarcinopsis* sp. (Figure 6a) and *Bracteacoccus* sp. (Figures 8b and 9a), which form green sheens in submerged environments. On the one hand, this could show that these genera are especially resistant in adverse conditions and capable of surviving in environments with chlorine and biocides; on the other hand, this could indicate that they are the first colonisers of the stone. Mats are usually found in submerged and amphibious environments and are typical of the presence of filamentous algae, especially cyanobacteria. *Phormidium*, *Leptolyngbya* (Figure 9c,d) and *Calothrix* are genera that are often the majority species in mats. On some fountains, these mats also have a high proportion of filamentous green algae, such as *Gongrosira* sp. and *Klebsormidium* sp. (Figure 9b). As for the mineral crusts, we found variability; they are usually made up of complex communities of various species of green algae and cyanobacteria (which sometimes form separate strata), but sometimes they are monospecific, made up of *Chlorosarcina* sp. and other indeterminate coccoid green algae.

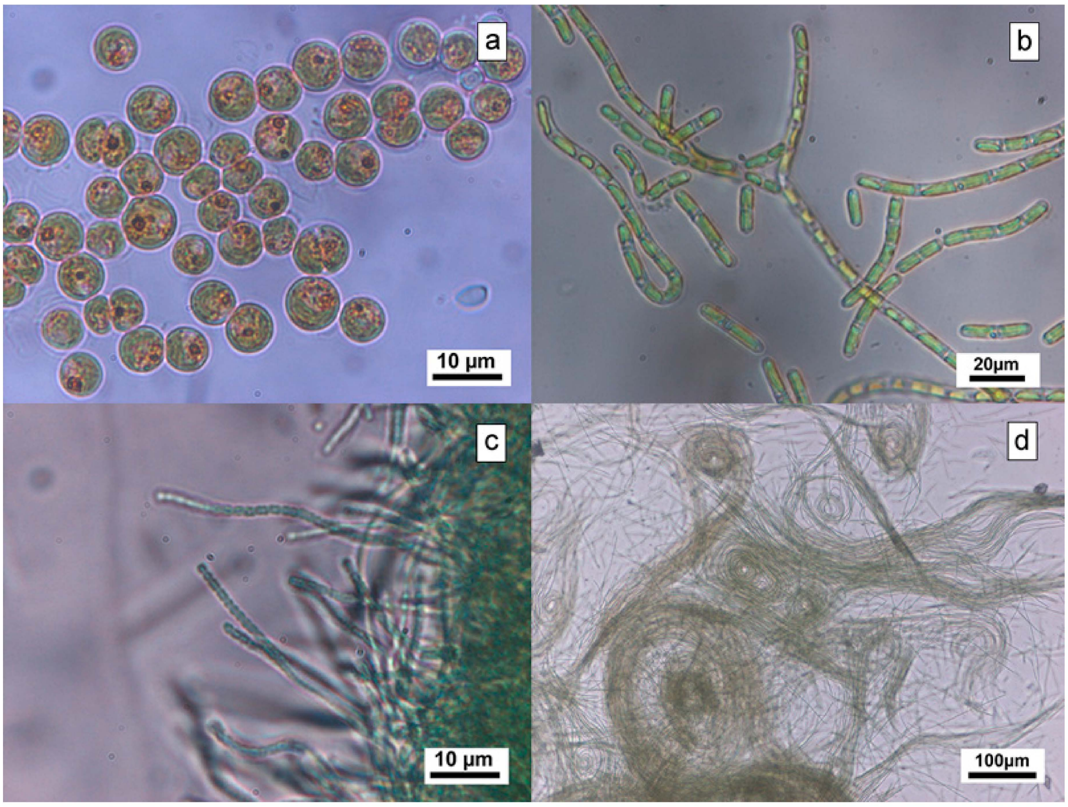

Figure 9. Photographs of the algal cultures *Bracteacoccus* sp. (**a**) and *Klebsormidium* sp. (**b**) and two different species of *Leptolyngbya* (**c**,**d**).

Most of the samples belong to amphibious or submerged environments. Only a few were taken in aerial zones far from water, although sometimes with high levels of relative humidity. In such zones, genera such as *Leptolyngbya*, *Klebsormidium*, *Stichococcus*, *Chlorococcum*, *Bracteacoccus*, *Cosmarium*, *Phormidium*, *Navicula* and *Chroococcopsis* were observed, forming coloured sheens and sometimes crusts. These same genera appear in other submerged and amphibious zones, so they are not exclusive to aerial environments. In the sampling points where samples were taken in different seasons, it was observed that there may be small variations between the observed species of algae, however the general composition is largely maintained and the predominant algae are usually the same. The small variations may be due to seasonal environmental factors or cleaning and maintenance work on the fountains.

If we compare the results with previous studies carried out on the fountains of the Alhambra [14,15,17,18], only the genera *Bracteacoccus*, *Klebsormidium*, *Choricystis*, *Stichococcus*, *Chroococcopsis*, *Cyanosarcina* and *Dichothrix* had never been recorded before. The rest of the observed microalgae had already been recorded in the past. It could be considered that new genera have appeared, but it has to be remembered that the same fountains have not always been sampled. In the thesis by Bolívar-Galiano [15] only seven fountains in the Nasrid Palaces were sampled, while in the study by Cuzman et al. [18] only samples from the Lindaraja fountain and the central fountain of the *Patio de la Sultana* were studied. In addition to some of the genera currently identified, in the previous works there are also some different ones. Specifically, we can observe a much greater number of species in the thesis by Bolívar-Galiano in 1994 [15], even though only the Nasrid Palaces' fountains were considered. One of the explanations for this phenomenon is that the presence of microalgae in these fountains was much greater 26 years ago, because today much more attention is paid to these microorganisms and the fountains undergo much stricter maintenance (Figure 10).

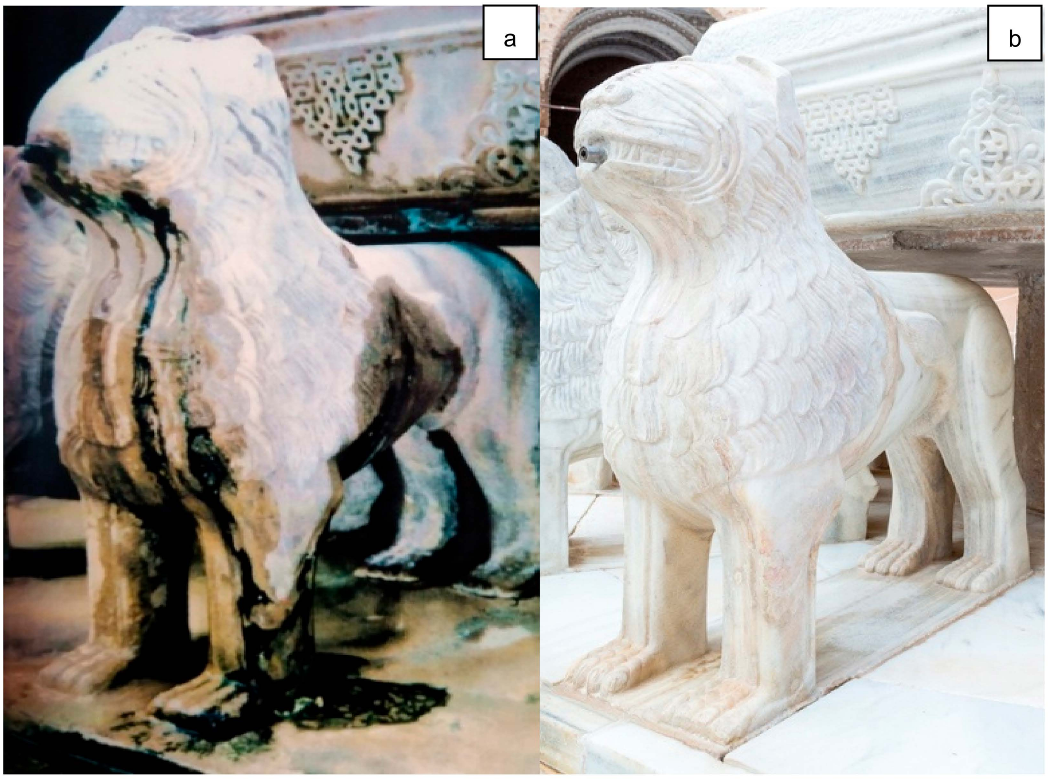

Figure 10. Presence of microalgae on one of the lions in the Fountain of the Lions: (**a**) an image from 1994 by F. Bolivar is shown on the left; (**b**) a modern image by G. Alfano in which one can see the effect of the cleaning treatment and lack of large sheens on the surface is shown on the right.

The genera we have identified are also common in other monumental fountains in Spain and Italy, where the following genera have been found: *Phormidium*, *Calothrix*, *Leptolyngbya*, *Lyngbya*, *Symploca*, *Schizothrix*, *Myxosarcina*, *Chamaesiphon*, *Chroococcidiopsis*, *Pleurocapsa*, *Chlorogloea*, *Pseudopleurococcus*, *Pleurastrum*, *Apatococcus*, *Chlorella*, *Chlorosarcina*, *Chlorosarcinopsis*, *Cosmarium*, *Chlorococcum*, *Scenedesmus*, *Stichococcus*, *Navicula*, *Nitzschia*, *Achnanthes* and *Cymbella* [18,24,25]. In addition to fountains, microalgae are also pioneers in colonising other stone monuments and are significant agents in their biodeterioration. In fact, algae and cyanobacteria are the majority components in the biofilms found in stone buildings in Europe, while cyanobacteria are the majority components in those found in Latin America [26]. In a study carried out on the most common green algae and cyanobacteria in monuments of different types of stone in the entire Mediterranean basin [27], all of the genera we found in the Alhambra were present. In fact, *Klebsormidium* sp., which did not appear in the fountains of the Alhambra in the past, is among the most common species in stone monuments. All of this information makes it clear that the microalgae growing in the Alhambra and Generalife are species that are very widespread and which are commonly found in stone monuments.

3.2. Obtaining Unialgal Cultures

Using some of the fresh samples, and before fixing them permanently, agar plates were inoculated and cultures were made in them to isolate the greatest possible number of microalgae species (Figure 11). The media used to isolate green algae were BBM (Phyto-Tech, Lenexa, KS, USA), while BG11 (Sigma-Aldrich, Madrid, Spain) media were used for cyanobacteria. From the beginning, the cultivation of diatoms was discarded, since they have greater nutritional demands and are a less common group. Finally, it was possible to isolate a total of 23 genera—10 green algae and 13 cyanobacteria (Table 3).

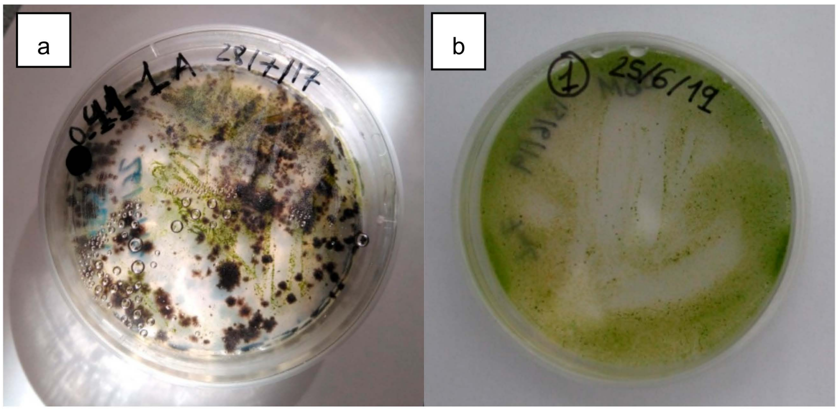

Figure 11. Microalgal cultures: (**a**) an initial culture using a sample is shown on the left; (**b**) a unialgal culture of *Neochloris* sp is shown on the right.

Table 3. Species obtained from unialgal cultures using samples collected in the Alhambra and Generalife. The fountain numbers (Tables 1 and 2) from which the cultures came are indicated in the left column.

Fountains	Green Algae	Fountains	Cyanobacteria
4	*Neochloris* sp.	2	*Dichothrix* sp.
14	*Stigeoclonium* sp.	4, 8, 15, 17	*Leptolyngbya* spp.
14, 8	*Chlorococcum* sp.	20, 7	*Chroococcopsis* sp.
9, 12, 16, 19	*Bracteacoccus* sp.	2, 8	*Pseudophormidium* sp.
4, 8, 14	*Scenedesmus* spp.	13	*Pseudanabaena* sp.
15	*Pseudopleurococcus* sp.	6, 21	*Calothrix* sp.
5, 10	*Klebsormidium* sp.	3	*Pleurocapsa* sp.
11, 13	*Choricystis* sp.	3	*Phormidium* sp.
3	*Apatococcus* sp.	5	*Cyanosarcina* sp.
4	*Gloeocystis* sp.	5	*Schizothrix* sp.
		5	*Nostoc* sp.
		5	*Chroococcidiopsis* sp.
		4	*Ammatoidea* sp.

The set of data is not completely representative of the site's microbiota, since it is known that the different species have different capacities for developing within in vitro cultures. Some species do not prosper in cultivation, even if they are predominant in the sample. Likewise, species that are not at all abundant in the samples may grow when cultivated, including species that are not observed in the sample, as has occurred with *Neochloris* sp., *Pseudanabaena* sp., some species of *Nostoc* sp., *Gloeocystis* sp. and *Leptolyngbya* sp. The latter genus grows very well when cultivated (Figure 9c,d), with up to 4 different strains having been isolated that are unidentified at the species level. This shows that there is a great diversity within this genus in the Alhambra and Generalife study areas. Moreover, in communities with great diversity it can be difficult to isolate different algae, especially filamentous cyanobacteria that form mats.

It is useful to have isolated cultures of species, firstly for identification, since the microalgae's life cycles can be observed, as well as for other different characteristics that are often difficult to appreciate in samples. Furthermore, through this process a culture collection of living algae has been initiated that may be used in future studies. All of the microalgae from the Alhambra and Generalife are to a greater or lesser extent resistant to the treatments currently used to eliminate them (for example, many are resistant to chlorine), which makes them very interesting for use in trials with new treatments aiming to halt the proliferation of these organisms in research projects currently in progress.

4. Conclusions

The fountains of the Alhambra and the Generalife are affected mainly by the growth of green algae, cyanobacteria and diatoms, which grow in spite of the physical and biocide treatments that are periodically carried out. Within these groups, the green algae are the most abundant, as is the case in many other examples of cultural heritage sites involving water and ornamental stone. In most cases different algae grow, forming complex communities and giving rise to sheens, mats or crusts. Coccoid unicellular green algae such as *Chlorococcum* sp., *Bracteacoccus* sp., *Chlorosarcina* sp. and *Chlorosarcinopsis* sp. are especially common, which sometimes form monospecific sheens. Other common genera of green algae are *Klebsormidium*, *Scenedesmus*, *Chlorella*, *Choricystis*, *Pleurastrum*, *Gongrosira*, *Leptosira* and *Pseudopleurococcus*. As for cyanobacteria, the most notable genera are *Pleurocapsa*, *Chamaesiphon*, *Chlorogloea*, *Chroococcidiopsis*, *Cyanosarcina*, *Myxosarcina* and *Chroococcopsis* (chroococcal); and *Phormidium*, *Calothrix*, *Leptolyngbya*, *Lyngbya* and *Schizothrix* (filamentous). The species of microalgae found here hardly vary with seasonal changes, and the most common microalgae are always the same ones. All of the genera found in this study correspond to widespread algae that are common in the colonisation of monumental fountains and stone cultural assets in general.

Ten green algae genera and thirteen cyanobacteria genera have been isolated in cultures (with different species of some of them), thereby initiating the creation of a collection of algal cultures from the Alhambra and Generalife. Although the identification of each species among them is still in progress, this will gradually be carried out as new observations are made in the cultures. This collection may be used in the future to carry out trials of new treatments or to study the synthesis of certain products using microalgae in the study area.

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