

Review

# The Marine Bacterial Genus *Euzebya* Is Distributed Worldwide in Terrestrial Environments: A Review

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**Abstract:** The advent of molecular tools, and particularly next-generation sequencing, has dramatically changed our knowledge of the diversity of microbial life on Earth. In recent decades, many studies on different terrestrial environments have described the intriguing diversity and abundance of *Euzebyales*/*Euzebyaceae*/*Euzebya*, yet its role in the geochemical cycle of elements is unknown. In addition, as far as we know, no *Euzebya* isolates have been obtained from terrestrial niches. In this review, it is shown that *Euzebya* and other haloalkaliphilic bacteria can thrive under harsh conditions, such as high concentrations of sodium and/or calcium, high electric conductivity and alkaline pH, highly variable temperatures, and water fluctuations. These conditions are quasi-extreme in the studied terrestrial environments. However, the culture media used so far for isolation have failed to reproduce the original conditions of these terrestrial ecosystems, and this is likely the reason why strains of *Euzebya* and other bacteria that inhabit the same niche could not be isolated. It is expected that culture media reproducing the environmental conditions outlined in this review could cope with the isolation of terrestrial *Euzebya* and other haloalkaliphilic genera.

**Keywords:** *Euzebya*; biofilms; caves; soils; rhizospheres; saline and hypersaline environments



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## 1. Introduction

In terms of microbial diversity, the oceans represent the largest biosphere habitat, containing about 70% of the prokaryotic biomass [1]. In recent decades, interest in the bioactive compounds from marine bacteria has grown enormously [2–8], and many works have focused on rare marine *Actinomycetota* [9–13]. One of the most intriguing and rare genera of marine *Actinomycetota* is *Euzebya*. No terrestrial *Euzebya* has been isolated so far.

The genus *Euzebya* was described by Kurahashi et al. [14] to accommodate a Gram-positive actinobacterial strain isolated from the epidermis of *Holothuria edulis*, a sea cucumber collected in the Sea of Japan. The strain was characterized by a reddish-orange or tangerine color and was able to grow in sodium chloride concentrations of 0.5–12%, but no growth was observed in the absence of sodium chloride or at a concentration of 15%. Optimal growth temperatures were in the range of 20–28 °C and pH 7–9. No growth was obtained at pH 6 or 10. The type strain is *Euzebya tangerina* from the new order *Euzebyales* and the new family *Euzebyaceae* [14]. A second member of the genus, *Euzebya rosea*, was isolated from the waters of the East China Sea and showed a light pink color, optimal growth at 25–30 °C, and pH 6–7. Optimal sodium chloride concentrations were 1–4% [15].

*Euzebya pacifica* was the third species of the genus, isolated from seawater collected at 150 m depth in the Eastern Pacific Ocean [16]. Colonies were pink, with optimal growth at

30–35 °C, in sodium chloride concentrations of 1–2%, and pH 6.5. This last species could grow in the absence of sodium chloride. The complete genome sequence of *E. pacifica* revealed its ecological roles in marine carbon, nitrogen, phosphorus, and sulfur cycles [17]. In general, the three marine species of *Euzebya* are characterized by their tolerance to relatively high sodium chloride concentrations, growth at neutral pH (7), and temperatures from 20 to 35 °C.

The advent of molecular tools, particularly next-generation sequencing (NGS), has dramatically changed the knowledge of the diversity of microbial life on Earth. In recent decades, many studies on different terrestrial environments, including caves [18–36], have described the diversity and abundance of *Euzebyales/Euzebyaceae/Euzebya*; however, as far as we know, no *Euzebya* isolates have been obtained from terrestrial niches. This prompted us to review the habitats where *Euzebya* sequences were found and their ecological requirements in order to understand the failures in the adoption of isolation protocols that led to the lack of terrestrial isolates. The diversity and abundance of *Euzebyales/Euzebyaceae/Euzebya* in most terrestrial environments are intriguing and its role in the geochemical cycle of elements is unknown, and thus the isolation of *Euzebya* strains could help to understand why this abundant bacterial genus is distributed worldwide, even in extreme environments.

## 2. Metagenomic Detection of *Euzebya* in the Environment: Caves

Caves are mineral environments, often oligotrophic in nature. Rocks, speleothems, and mineral deposits, such as moonmilk, are colonized by microbial communities, which develop as colored biofilms [18–20]. To our knowledge, the first report on the occurrence of *Euzebya* in caves was in a study by Cuezva et al. [18]. In Altamira Cave, Spain, sequences with 82–92% similarity to the nearest relative *Euzebya tangerina* were retrieved from grey biofilms, suggesting that they probably represented an unknown species. *Euzebya* represented 72.8% of the clones retrieved from the grey biofilms [18]. Riquelme et al. [19] recovered representatives of *Euzebyales* from colored microbial mats found in volcanic caves in the Azores, Hawai'i, and New Mexico, and stated that the different clades obtained suggested a significant diversity within the sequences found. Other papers reported *Euzebya* sequences from caves in different geographical regions [21–36] (Table 1).

The composition of microbial communities was found to be dependent on geochemical and microclimatic parameters. In this context, Frazier [29] reported the high relative abundance of *Euzebya* (up to 30.7%) in one cave and its negligible occurrence (up to 0.2%) in another cave from mineralogically similar formations located 65 km apart. The difference in abundance was attributed to flooding and clay deposition in the *Euzebya*-rich cave. Also interesting was the abundance of *Euzebyaceae* in pink biofilms from Pindal Cave, Spain (7.0–16.0%), coincident with the pink color of the three described *Euzebya* species [36].

A recent study was conducted on the biofilms present in Covadura Cave, located in the gypsum karst of Sorbas, Almeria, Spain. The karst comprises over 100 km of passages within the six most important caves (Covadura Cave, GEP Complex, C3 Cave, Gypsum Cave, Treasure Cave, and Water Cave), which are subject to condensation–dissolution mechanisms. Water condensation on the cooler walls of Covadura Cave takes place mainly during the dry period (July to October) and the biofilms show water droplets on their surface. Biofilm proliferation has been associated with the strong condensation existing in some caves [36], as condensation favors the colonization of cave walls by microorganisms [18,19].

The data revealed that *Euzebyaceae* were abundant in Covadura Cave white biofilms collected in 2010, but their relative abundance was drastically reduced in the 2022 sampling (Table 1). This could be associated with the severe droughts, the last of which occurred between 2017 and 2018, and which continue until now. In the yellow biofilms, the decrease in abundance was lower.

**Table 1.** Occurrence and relative abundance of *Euzebyales*/*Euzebyaceae*/*Euzebya* in caves.

| Cave                             | Abundance % | Taxa               | Type of Sample           | References         |
|----------------------------------|-------------|--------------------|--------------------------|--------------------|
| Altamira Cave, Spain             | n.a.        | <i>Euzebya</i>     | Grey biofilms            | [18]               |
| Volcanic caves, Portugal, USA    | n.a.        | <i>Euzebyales</i>  | Biofilms                 | [19]               |
| Hawai'i lava caves, USA          | n.a.        | <i>Euzebya</i>     | Biofilms                 | [20]               |
| Heshang Cave, China              | n.a.        | <i>Euzebya</i>     | Weathered rocks          | [21]               |
| Laugerie-Haute shelter, France   | 2.6 *       | <i>Euzebya</i>     | Biofilms                 | [22]               |
| Scarisora Ice Cave, Romania      | n.a.        | <i>Euzebya</i>     | Ice core                 | [23]               |
| Honda del Bejenado Cave, Spain   | 43.9 *      | <i>Euzebya</i>     | Yellow biofilms          | [24]               |
| B2 Cave, India                   | 1.16        | <i>Euzebyales</i>  | Cave wall                | [25]               |
| Honda del Bejenado Cave, Spain   | 31.7        | <i>Euzebya</i>     | Yellow biofilms          | [26]               |
| Fuente de la Canaria Cave, Spain | 0.2–2.7     | <i>Euzebya</i>     | Yellow biofilms/moonmilk | [26]               |
| Bucara II Cave, Spain            | 2.9–11.0    | <i>Euzebya</i>     | Pink deposit/moonmilk    | [26]               |
| Llano de los Caños Cave, Spain   | 3.0         | <i>Euzebya</i>     | Speleothem               | [26]               |
| Yixing Shanjuan Cave, China      | 4.2         | <i>Euzebya</i>     | Speleothem               | [27]               |
| Chimalacatepec lava tube, Mexico | 6.2         | <i>Euzebya</i>     | Stromatolite             | [28]               |
| RN5 Cave, USA                    | 30.7        | <i>Euzebya</i>     | Biofilms                 | [29]               |
| RN5 Cave, USA                    | 13.8        | <i>Euzebyaceae</i> | Biofilms                 | [29]               |
| KN14 Cave, USA                   | 0.1         | <i>Euzebya</i>     | Biofilms                 | [29]               |
| Royal Palm Cave, Galapagos       | n.a.        | <i>Euzebya</i>     | White biofilms           | [30]               |
| Pukzing Cave, India              | n.a.        | <i>Euzebya</i>     | Cave sediments           | [31]               |
| Altamira Cave, Spain             | 33–79       | <i>Euzebyaceae</i> | White biofilms           | [32]               |
| Altamira Cave, Spain             | 20–42       | <i>Euzebyaceae</i> | Light brown biofilms     | [32]               |
| Heshang Cave, China              | n.a.        | <i>Euzebya</i>     | Weathered rocks          | [33]               |
| Moon National Monument, USA      | n.a.        | <i>Euzebya</i>     | Biofilms/Speleothems     | [34]               |
| Geomunoreum lava tubes, Korea    | 0.3–0.7     | <i>Euzebya</i>     | Microbial mats           | [35]               |
| Pindal Cave, Spain               | 7.0–16.0    | <i>Euzebyaceae</i> | Pink biofilms            | [36]               |
| Covadura Cave, Spain             | 48.4–52.5   | <i>Euzebyaceae</i> | White biofilms (2010)    | Unpublished report |
|                                  | 0.1–21.7    | <i>Euzebyaceae</i> | White biofilms (2022)    |                    |
|                                  | 0.7–10.4    | <i>Euzebyaceae</i> | Yellow biofilms (2010)   |                    |
|                                  | 3.4–9.0     | <i>Euzebyaceae</i> | Yellow biofilms (2022)   |                    |

n.a., data not available. \* Abundance of clones.

*Euzebya* was also abundant in volcanic caves. The genus was found in caves in the Azores, Canary Islands, Galapagos, Hawai'i, Idaho, Tennessee, and Mexico [19,20,24,26,28–30,34]. Gonzalez-Pimentel et al. [24] stated that yellow biofilms from a cave on the Canary Island of La Palma were dominated by metabolically active *Euzebya* (43.9% RNA clones vs. 26.0% DNA clones). In the other three La Palma caves [26], the relative abundance of *Euzebya* was lower (Table 1).

La Palma caves are characterized by the leaching of dissolved organic matter from the soil surface, and its transport inside the cave is favored by the low thickness and high porosity of the volcanic rock [37]. *Euzebya* colonizes the mineral formations and deposits in caves, usually at an alkaline pH (8–10), where it develops as biofilms of different colors. The wide distribution in karstic, volcanic, and gypsum caves all over the world indicates that terrestrial *Euzebya* can thrive in these humid, largely mineral environments [38].

### 3. *Euzebyales* in Extreme Environments

Saline and hypersaline terrestrial environments include salt mines, sediments of desiccated salt lakes, saline and alkaline soils, salt marshes, etc. These environments often have salt concentrations higher than that of seawater and support halophilic microorganisms that have adapted to deal with extreme environmental parameters (high salt concentrations, temperatures, and pH), although their community composition and structure vary depending on salinity fluctuations in the environment [39].

The occurrence of *Euzebya* in these environments has been reported in numerous studies [40–55], denoting the ability of the members of this genus to prosper in habitats with high salt concentrations (Table 2).

Interestingly, the wide occurrence of *Euzebya* and its haloalkaliphilic relatives has been registered in the drained sediments of former Mexican lakes (Texcoco and Rincon de Parangueo) [40,45], and the Songnen Plain of Northeast China, one of the three regions with extensive saline–sodic soils in the world [50–53].

The Texcoco Lake sediments are characterized by a very high pH (10) and an electric conductivity (EC) of up to 179.8 dS m<sup>-1</sup> [40,42,44]. There, *Euzebya* was one of the dominant bacterial genera with relative abundances >10% [42]. It has been reported that organic carbon additions to the sediments increased *Euzebya* abundance [42].

In the Songnen Plain of Northeast China, with very high pH (>10) and high EC, *Euzebya* showed high relative abundance [50,52], as well as a high sodicity/salinity niche preference, however, the genus was depleted or absent when sodicity/salinity decreased [51].

In other highly saline environments, such as salt marshes, mines, and lake soils, *Euzebya* was detected in the rhizosphere of halophytic plants [41,43,47–49,54].

Chen et al. [56], using comparative genomics, investigated the salt adaptation mechanism of species within the class *Nitriliruptoria*, including *Euzebya tangerina* and *E. rosea*. They showed that the *Nitriliruptoria* species possess specific salt tolerance mechanisms.

Deserts, covering around 33% of the planet's surface, have been explored in search of extremophilic and extremotolerant *Actinomycetota* producing novel bioactive compounds [57–60]. However, the use of molecular tools (next-generation sequencing) revealed dominant members of the extremophilic microbial communities that have not been yet isolated. They included *Euzebya*, both in cold environments (Antarctica) and hot deserts (Atacama, Sahara, Colorado Plateau, etc.) [61–72] (Table 2).

**Table 2.** Occurrence of *Euzebyales*/*Euzebyaceae*/*Euzebya* in extreme environments.

| Location                            | Taxa              | Type of Sample   | References |
|-------------------------------------|-------------------|------------------|------------|
| Saline and hypersaline environments |                   |                  |            |
| Texcoco, Mexico                     | <i>Euzebya</i>    | Soil             | [40]       |
| Dafeng, Jiangsu, China              | <i>Euzebya</i>    | Soil/Rhizosphere | [41]       |
| Texcoco, Mexico                     | <i>Euzebya</i>    | Soil             | [42]       |
| Khewra salt mines, Pakistan         | <i>Euzebya</i>    | Soil/Rhizosphere | [43]       |
| Texcoco, Mexico                     | <i>Euzebya</i>    | Soil             | [44]       |
| Rincon de Parangueo, Mexico         | <i>Euzebyales</i> | Sediment         | [45]       |
| Rincon de Parangueo, Mexico         | <i>Euzebya</i>    | Sediment         | [46]       |
| Chaka Salt Lake, China              | <i>Euzebya</i>    | Rhizosphere      | [47]       |
| Jeddah, Saudi Arabia                | <i>Euzebya</i>    | Soil/Rhizosphere | [48]       |
| Lebrija, Seville, Spain             | <i>Euzebya</i>    | Soil/Rhizosphere | [49]       |
| Songnen Plain, Jilin, China         | <i>Euzebya</i>    | Soil             | [50]       |

Table 2. Cont.

| Location                                | Taxa               | Type of Sample    | References |
|---|--------------------|-------------------|------------|
| Songnen Plain, Jilin, China             | <i>Euzebya</i>     | Soil              | [51]       |
| Songnen Plain, Jilin, China             | <i>Euzebya</i>     | Soil              | [52]       |
| Songnen Plain, Jilin, China             | <i>Euzebya</i>     | Soil              | [53]       |
| Daqing, Heilongjiang, China             | <i>Euzebya</i>     | Soil/Rhizosphere  | [54]       |
| Hisar, Haryana, India                   | <i>Euzebya</i>     | Soil              | [55]       |
| Cold and hot deserts                    |                    |                   |            |
| Victoria Valley, Antarctica             | <i>Euzebya</i>     | Rocks             | [58]       |
| Atacama Desert                          | <i>Euzebya</i>     | Rocks             | [59]       |
| McMurdo Dry Valleys, Antarctica         | <i>Euzebyales</i>  | Soil              | [60]       |
| McMurdo Dry Valleys, Antarctica         | <i>Euzebyaceae</i> | Soil              | [61]       |
| Australia/Northern Antarctica           | <i>Euzebya</i>     | Soil              | [62]       |
| Cabo de Gata, Nijar Natural Park, Spain | <i>Euzebyaceae</i> | Soil              | [63]       |
| North-Central Algeria                   | <i>Euzebya</i>     | Soil/Rhizosphere  | [64]       |
| Eastern Pamir, Tajikistan               | <i>Euzebyaceae</i> | Rocks/soil crusts | [65]       |
| Mackay Glacier region, Antarctica       | <i>Euzebyales</i>  | Soils             | [66]       |
| Colorado Plateau Desert, USA            | <i>Euzebya</i>     | Soil              | [67]       |
| Northwest deserts of China              | <i>Euzebya</i>     | Soil              | [68]       |
| Gurbantunggut Desert, Xinjiang, China   | <i>Euzebya</i>     | Soil/Rhizosphere  | [69]       |

*Euzebya* was one of the most frequently detected genera in Australian and Northern Antarctica soils. There, *Actinomycetota* diversity increased with increasing pH and sodium concentration, and this applies particularly to *Euzebya* [65].

The McMurdo Dry Valleys is the largest ice-free soil region in Antarctica. There, *Euzebyales* were abundant only in the soil samples with moisture below 6.82% but largely declined or were absent in the soil with moisture content above 15.57% [63]. In Victoria Valley, within McMurdo Dry Valleys, two families, *Euzebyaceae* and *Rubrobacteraceae*, were abundant (over 30%) in endolithic niches and less frequent in soils. It has been reported that water availability largely conditioned the distribution of these actinobacterial families [64].

In Antarctica, the rock-inhabiting microbial communities are dominated by *Cyanobacteria* and *Actinobacteria*, particularly *Euzebya*, which also occupy hypolithic niches [63]. In the cold mountain desert of Eastern Pamir in Tajikistan, *Euzebya* was present both in rocks (endolithic) and in biological soil crusts [68].

In addition, *Euzebya* was found in endolithic niches in the hyperarid zone of the Atacama Desert, Chile [62], and in soils from the Sahara, Colorado Plateau, and China [67,70–72].

#### 4. *Euzebyales* in Soils and Other Diverse Environments

Table 3 shows the occurrence of *Euzebya* in agricultural and contaminated soils, and other diverse environments [73–119].

*Euzebya* was found in the rhizosphere of *Agave lechuguilla* in the saline and oligotrophic soils of Cuatro Ciénegas Basin, Mexico [76,81], as well as in other plant rhizospheres from different regions [73,75,79,81,85,91,92,95].

**Table 3.** Occurrence of *Euzebyales*/*Euzebyaceae*/*Euzebya* in soils and other diverse environments.

| Location  | Taxa               | Type of Sample    | References |
|---|--------------------|-------------------|------------|
| Soils   |                    |                   |            |
| Unknown, China                                    | <i>Euzebya</i>     | Soil/Rhizosphere  | [73]       |
| Valle del Yaqui, México                           | <i>Euzebya</i>     | Soil              | [74]       |
| Unknown, Korea                                    | <i>Euzebyaceae</i> | Soil/Rhizosphere  | [75]       |
| Cuatro Ciénegas Basin, Mexico                     | <i>Euzebya</i>     | Soil/Rhizosphere  | [76]       |
| Lublin region, Poland                             | <i>Euzebyaceae</i> | Soil              | [77]       |
| Beijing, China                                    | <i>Euzebya</i>     | Soil              | [78]       |
| Dingxi, China                                     | <i>Euzebya</i>     | Rhizosphere       | [79]       |
| Fogo Island, Cape Verde                           | <i>Euzebya</i>     | Volcanic soils    | [80]       |
| Cuatro Ciénegas Basin, Mexico                     | <i>Euzebya</i>     | Soil/Rhizosphere  | [81]       |
| Hotan City, Xinjiang Uygur, China                 | <i>Euzebya</i>     | Soil              | [82]       |
| Pernambuco, Brazil                                | <i>Euzebyaceae</i> | Soil              | [83]       |
| Outside a sabkha, Abu Dhabi                       | <i>Euzebyales</i>  | Soil              | [84]       |
| Qapqal County, Xinjiang, China                    | <i>Euzebyaceae</i> | Soil/Rhizosphere  | [85]       |
| Zaragoza, Spain                                   | <i>Euzebya</i>     | Soil              | [86]       |
| Weizhou and Xieyang Islands, China                | <i>Euzebya</i>     | Volcanic soils    | [87]       |
| Springfield Farm, Western Cape, South Africa      | <i>Euzebya</i>     | Peatlands         | [88]       |
| Omaha/Lincoln, Nebraska, USA                      | <i>Euzebya</i>     | Soil              | [89]       |
| International Centre for Insect Physiology, Kenya | <i>Euzebyaceae</i> | Soil              | [90]       |
| Sugarcane Research Institute, Nanning, China,     | <i>Euzebya</i>     | Soil/Rhizosphere  | [91]       |
| Hetao Ningxia Plain, China                        | <i>Euzebya</i>     | Soil/Rhizosphere  | [92]       |
| Different Russian regions and Antarctica          | <i>Euzebya</i>     | Soils             | [93]       |
| Saline–alkali regions, Northeastern China         | <i>Euzebya</i>     | Soil              | [94]       |
| Jinzhong City, Shanxi Province, China             | <i>Euzebya</i>     | Soil/Rhizosphere  | [95]       |
| Contaminated soils                                |                    |                   |            |
| Daqing oilfield, China                            | <i>Euzebya</i>     | Oil polluted soil | [96]       |
| Kuwait  | <i>Euzebya</i>     | Oil polluted soil | [97]       |
| Shandong Province, China                          | <i>Euzebya</i>     | Bauxite residue   | [98]       |
| Northwest region of Bangladesh                    | <i>Euzebya</i>     | As-polluted soil  | [99]       |
| Copper mine, Miami, Arizona, USA                  | <i>Euzebyaceae</i> | Mine waste rock   | [100]      |
| Xinjiang, Northwest China                         | <i>Euzebya</i>     | U repositories    | [101]      |
| Diverse environments                              |                    |                   |            |
| Mausoleo Cuadrangular, Carmona, Spain             | <i>Euzebya</i>     | Roman tomb        | [102]      |
| Cambodian aquifer                                 | <i>Euzebya</i>     | As-rich sediments | [103]      |
| EsTrenc saltern, Mallorca, Spain                  | <i>Euzebya</i>     | Brine/Rhizosphere | [104]      |
| Akkol salt lake, Russia                           | <i>Euzebyaceae</i> | Lakeshore         | [105]      |
| Pozo de la Higuera, Almeria, Spain                | <i>Euzebya</i>     | Irrigation waters | [106]      |
| Houssaye Point, Erquy, France                     | <i>Euzebya</i>     | Marine lichens    | [107]      |
| Wawel Royal Castle, Krakow, Poland                | <i>Euzebyaceae</i> | Building stones   | [108]      |
| Potter Cove, Antarctica                           | <i>Euzebya</i>     | Seawater          | [109]      |

Table 3. Cont.

| Location                            | Taxa           | Type of Sample      | References |
|-------------------------------------|----------------|---------------------|------------|
| Bentonite deposit, Almeria, Spain   | <i>Euzebya</i> | Bentonite           | [110]      |
| Coral reefs, Lakshadweep, India     | <i>Euzebya</i> | Demosponges         | [111]      |
| Dogs, USA                           | <i>Euzebya</i> | Feces               | [112]      |
| Coastal waters, Yantai City, China, | <i>Euzebya</i> | Bacterioplankton    | [113]      |
| Dogs, USA                           | <i>Euzebya</i> | Vomit               | [114]      |
| Dogs, USA                           | <i>Euzebya</i> | Oral cavity         | [115]      |
| Human microbiome                    | <i>Euzebya</i> | Urogenital          | [116]      |
| Human microbiome                    | <i>Euzebya</i> | Oral cavity         | [117]      |
| Human microbiome                    | <i>Euzebya</i> | Cerebrospinal fluid | [118]      |
| West coast of India                 | <i>Euzebya</i> | Seawater            | [119]      |

*Euzebya* is represented in soils all over the world [74,77,78,80,82–84,86,89,90,93,94]. Several authors have reported the occurrence of *Euzebya* in clean and healthy soils and its absence in polluted soils [77,96,97]. However, *Euzebya* has also been found in bauxite residue disposal areas and copper mine wastes [98–101].

The presence of *Euzebya* has been recorded in saltern and salt lakes, terrestrial and sea waters, marine organisms [103–107,111,113,119], bentonite [110], animals [112,114,115], and humans [116–118]. In addition, the genus was found on a sandstone surface, covered by efflorescences, at the Wawel Royal Castle in Poland [108]. In a report on the microbial communities of efflorescences from Roman tombs in the Carmona Necropolis, Spain, a high number (60%) of clones showing a similarity of 87% with the genus *Nitriliruptor* were retrieved. At the time of this study, 2009 [102], no *Euzebya* species had been described. However, a further study [26] showed that these clones had 92.5% similarity with *Euzebya tangerina*, while an updated revision of these clones showed a similarity of 91.7–92.7% with *Euzebya pacifica*.

### 5. Relationship of *Euzebyales* with Other Members of Microbial Communities in Diverse Environments

A review of all the reports available in the literature provided some insights into the relationship of *Euzebya* with other taxa in different environments. In fact, several taxonomic groups may inhabit the same niche as *Euzebya*. Thus, *Euzebya* is present in most caves together with *Crossiella*, *Rubrobacter*, wb1-P19 (*Nitrosococcales*), and *Gaiella*, among other genera [19,21,23,27–29,33–36]. Caves are characterized by high relative humidity, in most cases near saturation, high mineral concentration, mainly of calcite in karstic and basaltic rocks in volcanic caves, as well as alkaline pH. In some pristine caves, oligotrophy is an environmental constraint.

In saline and hypersaline environments, the order *Euzebyales* is accompanied by other orders common to these extreme environments, such as *Nitriliruptorales*, *Rubrobacterales*, *Solirubrobacterales*, *Gaiellales*, *Acidimicrobiales*, *Oceanospirillales*, *Rhizobiales*, KSA1 (*Bacteroidetes*), etc. [41,42,45,49–53]. Most members of these orders require high pH and salt concentrations, and oligotrophy is common in these environments. There are a few outstanding reviews on heavy metal resistance in halophilic *Bacteria* and *Archaea* [120–122]. These and other studies [123,124] revealed that the mechanism of heavy metal resistance is associated with salinity tolerance and an increase in salinity and pH-enhanced tolerance to toxic metals. *Nitriliruptoria* species possess salt tolerance mechanisms [56] and it is likely that heavy metal resistance could also be attributed to *Euzebya*.

In deserts, *Euzebya* has been found together with *Nitriliruptor*, *Rubrobacter*, *Solirubrobacter*, *Gaiella*, *Halomonas*, etc. [62,64,65,68–71]. Water availability is scarce in deserts and environmental conditions become more challenging (e.g., strong oligotrophy and high

mineral deposits). Most of these genera are known for their ability to resist extreme desiccation, high UV and ionizing radiation, temperature fluctuations, and high salinity and metal concentrations [62]. In addition, the pink color of most species of these genera is due to the presence of bacterioruberin, a carotenoid pigment with UV-protective properties. It is suggested that *Euzebya* may possess most of the above-described characteristics, although as far as we know no pigment identification has been reported for any of the three marine species.

In soils, the co-occurrence of *Euzebyales* with *Nitriliruptorales*, *Rubrobacterales*, *Solirubrobacterales*, *Gaiellales*, *Oceanospirillales*, *Rhizobiales*, etc., is frequently reported [84,86–89], as previously stated for caves, saline, hypersaline, and desert environments.

To summarize, some microbial lineages present in harsh terrestrial environments show successful adaptation strategies and the ability to cope with available scarce nutrient sources in unfavorable climatic and geochemical conditions.

## 6. Culture Media for the Isolation of *Euzebya* in Terrestrial Environments

From 90 papers reporting the occurrence of *Euzebya* in different environments (Tables 1–3), only 13 described the isolation of bacteria, but only three marine species of *Euzebya* were successfully isolated on marine culture medium with 1–2% of sodium chloride, pH near 7, and at a temperature of 25 °C [14–16].

The terrestrial environments where *Euzebya* have been found are characterized by haloalkaliphilic conditions, high pH (9–10), and high to moderate salt contents. The availability of water in these ecosystems is widely variable, from dry conditions to 100% relative humidity, which suggests the great adaptability of this genus. In addition, the range of mean temperatures of these environments is highly variable, from –30 °C (winter in McMurdo Dry Valleys) to >40 °C in deserts, with large daily temperature fluctuations in each location.

The culture media used by different authors contained a wide array of carbon and nitrogen sources (peptone, tryptone, starch, tyrosine, glycerol, asparagine, sodium caseinate, malt extract, humic acid, glucose, oatmeal, etc.), mainly used for the isolation of *Actinomycetota*. At the same time, the media rarely contained high concentrations of salts (sodium or calcium), and the pH was not adjusted to the alkalinity ranges where terrestrial *Euzebya* and other related bacteria are abundant. None of these attempts were able to isolate strains of *Euzebya*, *Nitriliruptor*, *Rubrobacter*, *Solirubrobacter*, *Gaiella*, *Halomonas*, etc., which clearly indicates that the culture media used failed to reproduce the ecological conditions where these bacteria succeed.

As a matter of fact, *Rubrobacter* strains were isolated from biodeteriorated Roman tombs and a church with abundant efflorescences using media developed for halobacteria (DSMZ media 372, 1018, 1350) [125]. Three of these strains represented a new species, *Rubrobacter braccarensis*, that grew on tryptone soya agar (TSA) with concentrations of 30% NaCl, with an optimum at 3–10%, in contrast to other described species of *Rubrobacter*, which did not grow at concentrations above 5–6% NaCl [126]. This proves that culture media mimicking the original ecological conditions can provide novel, not yet cultured strains.

## 7. Attempts to Isolate *Euzebya* from Pindal Cave

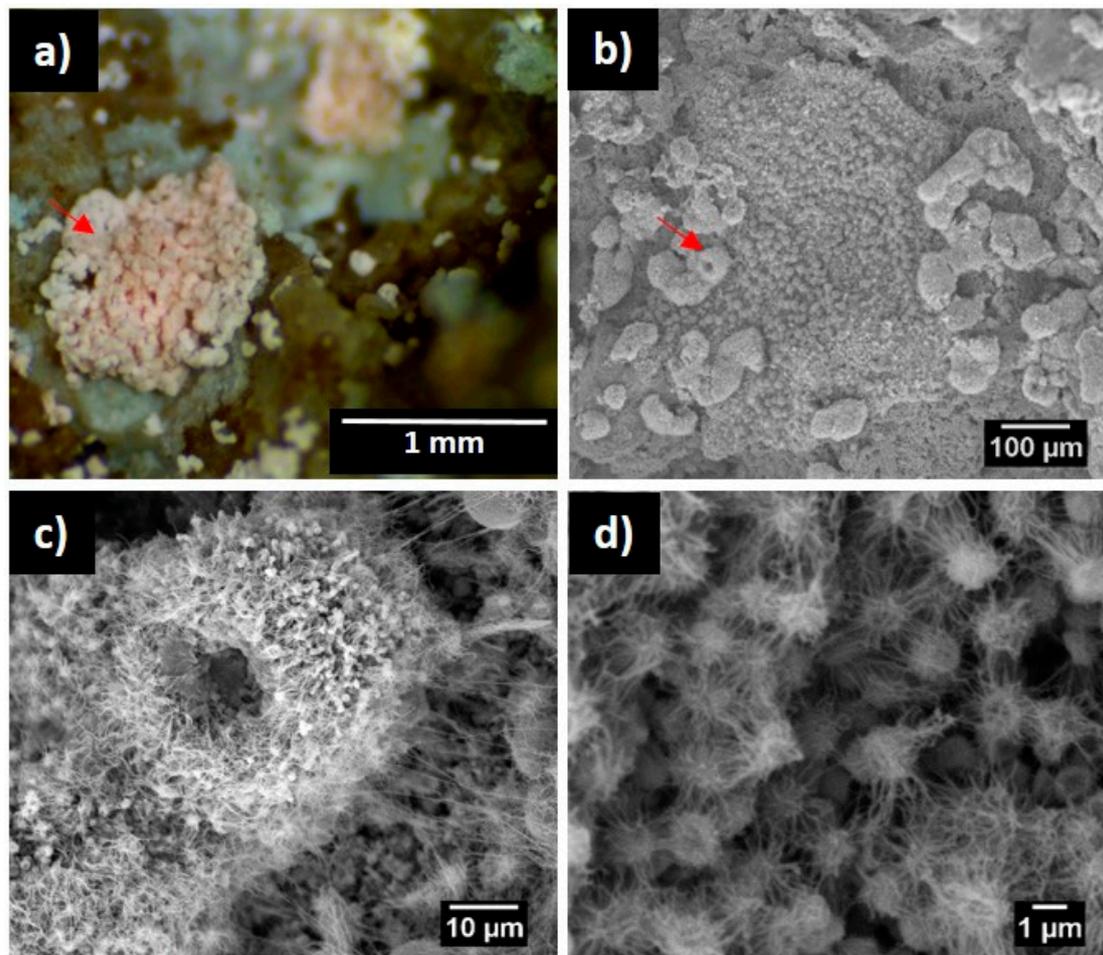
Pindal Cave is a shallow limestone cave formed through epigenic processes and located very close to the surface. The cave is 590 m long and due to the geographical location has a humid oceanic climate. The cave has a stable annual temperature (11.6 °C) with only minor fluctuations throughout the year (<2 °C/year). This cave is well-ventilated with relatively low annual average values of CO<sub>2</sub> (680 ppm) and radon (950 Bq/m<sup>3</sup>) [36].

In Pindal Cave, pink biofilms primarily develop on the surface of calcite speleothems in areas near the entrance and *Euzebyaceae* reached a relative abundance of 7–16% (Table 4); the biofilms have a rough surface and are formed by aggregates of cells, mostly rounded, with extensive filaments (Figure 1). Other abundant genera were *Crossiella* and wp1-P19. The ecological significance of the five top taxa in Pindal Cave was discussed elsewhere [36,127,128].

However, attempts to isolate *Euzebya* using different culture media failed. The following media were used: nutrient agar (NA), B-4 medium [129], GYM *Streptomyces* medium (DSMZ 65), Dimethylsulfone medium [130], TSA, diluted TSA/1000, and TSA supplemented with NaCl (3%) and MgSO<sub>4</sub>·7H<sub>2</sub>O (2%) (DSMZ 1350) [125]. In all these media, the pH was near 7, and not as markedly alkaline as *Euzebya* requires (pH 9–10), as denoted by their habitats; in other cases, the absence of relatively high NaCl concentrations likely prevented its isolation.

**Table 4.** NGS relative abundances (%) of the top five taxa in samples from pink biofilms, Pindal Cave, Spain [36].

| Genus              | Sample Bal1 | Sample Bal4 |
|--------------------|-------------|-------------|
| <i>Euzebyaceae</i> | 16          | 7           |
| <i>Crossiella</i>  | 8           | 7           |
| wb1-P19            | 5           | 11          |
| PLTA13             | 4           | 5           |
| <i>Nitrospira</i>  | 3           | 3           |
| Other genera       | 64          | 67          |



**Figure 1.** (a) Pink biofilms growing on calcite speleothems in Pindal Cave, Spain (red arrow). (b–d) Scanning electron microscopy microphotographs of pink biofilms from Pindal Cave. (b) General view of pink biofilms (red arrow). (c,d) Bacterial filaments forming the biofilm.

A culture-dependent approach revealed 33 isolates representing 19 identified species, as listed in Table 5. The isolates were affiliated with the phyla *Actinomycetota* (three genera) and *Bacillota* (five genera). Both phyla are widespread in nature [131–134] and the genera isolated from pink biofilms were spore-forming bacteria. Remarkably, none of the bacteria isolated were representatives of the top major taxa recovered by NGS (Table 4). In fact, NGS of the pink biofilm revealed only *Streptomyces* with a relative abundance of 0.01%, which was also isolated on culture media. The results indicated significant differences in the bacterial communities detected in the pink biofilm when applying these two different approaches.

**Table 5.** Strains isolated from pink biofilms, Pindal Cave, Spain.

| Strain | Culture Medium          | Identification (% Similarity) *             | Accession Number |
|--------|-------------------------|---|------------------|
| R2-11  | B-4                     | <i>Peribacillus frigoritolerans</i> (100)   | OR037511         |
| R2-14  | Dimethylsulfone         | <i>Streptomyces</i> sp. (99.89)             | OR037512         |
| R2-15A | TSA/1000                | <i>Streptomyces</i> sp. (99.66)             | OR037513         |
| R2-15B | TSA/1000                | <i>Micromonospora chalcea</i> (99.77)       | OR037514         |
| R2-1   | NA                      | <i>Bacillus altitudinis</i> (99.89)         | OR037515         |
| R2-5   | TSA                     | <i>Bacillus thuringiensis</i> (100)         | OR037516         |
| R2-6   | TSA                     | <i>Bacillus mojavensis</i> (100)            | OR037517         |
| R2-8   | GYM <i>Streptomyces</i> | <i>Psychrobacillus vulpis</i> (98.92)       | OR037518         |
| R4-12  | B-4                     | <i>Paenibacillus peoriae</i> (99.78)        | OR037519         |
| R4-13  | TSA/1000                | <i>Peribacillus frigoritolerans</i> (100)   | OR037520         |
| R4-14  | TSA/1000                | <i>Streptomyces</i> sp. (99.67)             | OR037521         |
| R4-15  | Dimethylsulfone         | <i>Rhodococcus erythropolis</i> (100)       | OR037522         |
| R4-17  | Dimethylsulfone         | <i>Rhodococcus koreensis</i> (99.31)        | OR037523         |
| R4-3   | GYM <i>Streptomyces</i> | <i>Psychrobacillus glaciei</i> (99.77)      | OR037524         |
| R4-4   | NA                      | <i>Paenibacillus pabuli</i> (99.78)         | OR037525         |
| R4-5   | NA                      | <i>Bacillus thuringiensis</i> (100)         | OR037526         |
| R4-6   | TSA                     | <i>Psychrobacillus vulpis</i> (98.92)       | OR037527         |
| R4-7   | TSA                     | <i>Peribacillus</i> sp. (99.03)             | OR037528         |
| R4-8   | TSA                     | <i>Metabacillus sediminilitoris</i> (99.03) | OR037529         |

\* Closest relative obtained by comparison with the NCBI database.

The inability of culture-dependent and independent methods to detect the same bacterial species has already been reported [135,136]. Laiz et al. [137] stated that culture-dependent techniques lead to an overestimation of the spore-forming bacteria, as shown in Table 5. They found that the apparent abundance of these bacteria can be explained by the fast growth of spores in the plates.

The failure in obtaining *Euzebya* isolates prompted us to review the ecological niches where *Euzebya* can be found. The survey revealed that the environmental conditions requested by *Euzebya* were not met in the culture media used thus far.

Culture media reproducing the environmental conditions outlined in this review, e.g., marine agar and SN medium (including 1/10 dilutions of these media), pH 9–10, and sodium chloride concentrations around 3% or more, could allow the isolation of terrestrial *Euzebya* and other haloalkaliphilic genera. Marine agar and SN medium [138] have been used for the isolation of marine *Euzebya* [14–16]. Alternatively, for maintaining a high pH, the medium Z8-NK, as described by Flores et al. [139], R2A, and/or other media with the addition of trace elements, amino acids, vitamins, and simple carbon sources to a minimal culture medium should be explored.

## 8. Concluding Remarks

NGS technologies have allowed the detection of unknown microorganisms and extended our knowledge of the diversity of microbial life on Earth. However, the majority of taxa are part of the yet-uncultured microbial dark matter that significantly contributes to ecosystem functioning [140,141].

The data (Tables 1–3) indicate that *Euzebya* is present across the entire biosphere. The question is whether their species were dispersed from marine sources to the terrestrial environment or if they are truly terrestrial, not yet described, species.

In this review, it is shown that *Euzebya* and other bacteria can thrive under harsh conditions, such as high concentrations of sodium and/or calcium, high electric conductivity, alkaline pH, and highly variable temperature and water fluctuations. These ecological conditions in the studied terrestrial environments are quasi-extreme.

Unfortunately, the culture media used so far for the isolation of *Euzebya* failed to reproduce the original conditions of these harsh ecosystems and this could be the reason why strains of *Euzebya* and other bacteria that inhabit the same niche were not isolated.

This review presents some of the pitfalls and limitations of commonly used culture media and suggests possible solutions to challenges faced in isolating terrestrial *Euzebya* strains. The importance of combining high-throughput sequencing and cultivation techniques is of the utmost interest for this task. Data on the physicochemical and environmental parameters of the terrestrial ecosystems where *Euzebya* thrives should be taken into account when designing appropriate culture media.

It is expected that the interest in the biogeochemical role and geographical distribution of *Euzebya* will promote the optimization of culture media, and in this way, researchers will be able to isolate novel *Euzebya* species from different terrestrial environments.

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