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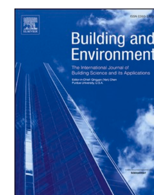

Laboratory development of subaerial biofilms commonly found on buildings. A methodological review

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A B S T R A C T

Study of the biodeterioration of building materials is often hampered by the inaccessibility of the colonization and impossibility of sampling in protected buildings considered part of the cultural heritage. There is therefore a recognised need to develop a laboratory mesocosm that realistically represents nature and enables the study and analysis of both the substrate and the organisms involved. Although many studies have investigated the formation of biofilms on various building materials, there is a lack of homogeneity and consensus in the methods and protocols used, which hampers comparison of the results obtained. This review aims to identify the different methods reported in the scientific literature, to organise these according to different factors (water access and types of organism and substrata used) and thus provide the research community with a guide for selecting the most appropriate methods according to the different objectives.

1. Introduction

Natural stone, wood and man-made materials such as concrete, mortars, brick, ceramic and glass are the main materials used in architecture and building construction, both in the past and at present [1]. Buildings and structures are ephemeral and finite, owing to the constant exposure of the materials to the environment. In recent years, events related to climate change have accelerated the weathering of materials, making both new construction and cultural heritage buildings increasingly threatened. For this reason, although many of these structures have survived over the ages, the increased vulnerability has led to growing concern regarding their conservation [2–4].

To develop conservation strategies, it is essential to understand the physical-chemical and biological processes that mediate the weathering of materials, how they work and the extent to which they can be tackled. Studying biological weathering processes involve numerous factors related to the organisms, substrate, surrounding environment and the interactions between these. Different organisms respond in different ways to changes in the environment, and their effect on the substrata, whether harmful or protective, is the consequence of a larger network of interactions [2,5,6].

Microbial communities that grow on exposed substrates, such as the surfaces of built cultural heritage, are known as subaerial biofilms (SABs). These are ubiquitous, self-sustaining microbial ecosystems that can be found on buildings, monuments and bare rock at all latitudes where direct contact with the atmosphere and solar radiation occurs. Subaerial biofilms are commonly defined as sessile microbial consortia established in a three-dimensional structure. They consist of multicellular communities of prokaryotic and/or eukaryotic cells embedded in a matrix that is at least partly composed of material synthesized by the microbial community [7–9]. Biofilm formation is a multi-step process that starts with microbial adhesion and the subsequent production and accumulation of an extracellular matrix composed of one or more polymeric substances such as proteins, polysaccharides, humic substances, extracellular DNA and sometimes other molecules such as those involved in cell-to-cell communication [10]. It is a complex process, which occurs over time on different materials and under different conditions.

Biofilm formation is highly dependent on environmental conditions, mainly water availability [11], but other factors such as type, orientation, substrate inclination and building architecture are also important [12]. All of these factors influence the likelihood of biofilm development, as well as the diversity and composition of the biofilms [5,13,14]. In particular, the structure of the building and the different architectural elements determine which areas of the building are more likely to become wet and consequently colonized. Access of water to the substrate via different pathways determines where water will be present in or on the walls and influences colonization [15]. Fig. 1 shows, in

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overview, the different water pathways related to the building architecture. Rainwater and fog can lead to accumulation of water on horizontal surfaces such as terraces (Fig. 1; 1) but also on other structures such as balconies, due to dripping from other elements (Fig. 1; 2). Water from the wet ground (Fig. 1; 3) can rise by capillary action, wetting the lower part of the walls [16]. In this case, the building material plays a fundamental role, as the physical properties (porosity, cracks, fissures, etc.) determine the height that the moisture line reaches [17]. Rainwater can also affect non-horizontal building surfaces and runoff through preferential water flow in some areas (Fig. 1; 4). or directly by wind-driven rainfall reaching the wall (Fig. 1; 5). In both cases, water will run off on vertical or inclined surfaces by gravity, draining down the wall. Thus, biofilm development will be probably more restricted by anchorage of the organisms than by water availability. In such cases, organisms with a greater capacity to produce matrix and adhere will be able to resist the flushing action of water and to grow and form biofilms.

Because of the multiple factors involved in biofilm formation, studies on outdoor biocolonization of buildings should preferably be carried out on site, directly on the buildings. Case studies have become popular as they have the advantage of enabling study of natural complex microbial communities that are well-structured and well adapted to the environment. Nonetheless, case studies have some major drawbacks: i) sampling restrictions aimed at preventing damage to the buildings, especially cultural heritage elements and ii) the impossibility of comparing the results of different studies owing to the large number of different factors involved in each building; laboratory studies are preferred in such situations.

Alternatively, laboratory studies can be carried out with modifiable, analyzable and destructible substrates, and they allow reproducible testing of different materials, organisms and treatments, without concern for preservation of the substrates. Moreover, environmental variables can be controlled in laboratory studies, and shorter generation times can be favoured, thus enhancing the growth of organisms under optimal conditions for development [18]. The use of organisms commonly found on built cultural heritage, or even model species, facilitates the study of all of the processes involved in biofilm formation. However, techniques or protocols for laboratory cultivation of biofilm communities on building materials, including the correct adhesion to the substrate and the production of extracellular matrix, have not yet been standardized. In order to develop specific protocols for developing biofilms, the building structure and the preferential water pathways must be taken into account (see Fig. 1).

The present review has been conducted in the light of the need to create complex model ecosystems in the laboratory. It aims to bring together studies in which subaerial biofilms have been successfully formed on different building materials, with different methods of supplying water. To this respect, the methodologies developed in the laboratory have been divided into two main groups according to water supply: *Static Mechanisms* and *Dynamic Mechanisms*. *Static Mechanisms* (SM) include mechanisms that simulate natural biofilm formation on horizontal surfaces where water does not exert a drag force and can reach the material either through the air (Fig. 1; 1 and 2) (water sprayed on a horizontal surface) or through capillary action (Fig. 1; 3) (water rising from bottom to top through the substrate), or even a combination of both. *Dynamic Mechanisms* (DM) include mechanisms that simulate the formation of biofilms on both inclined and vertical surfaces exposed to rain indirectly (Fig. 1; 4) or directly (Fig. 1; 5) and where run-off occurs [19].

2. Organisms used in laboratory experiments to induce subaerial biofilms on building materials

The microbial communities that make up subaerial biofilms (SABs) comprise phototrophic algae and cyanobacteria, heterotrophic fungi and bacteria. There are three alternatives to consider when selecting which organisms to use in laboratory experiments: i) use of model monospecies cultures], ii) use of ubiquitous monospecies culture [20, 21] and iii) use of multispecies cultures obtained from natural biofilms [22,23]. Some of the species used are model species in many disciplines, and information about their physiology or metabolism is therefore available (E.g. *Nostoc* sp. [24]). Others are ubiquitous in cultural heritage or native to the region studied, enabling more accurate analysis of

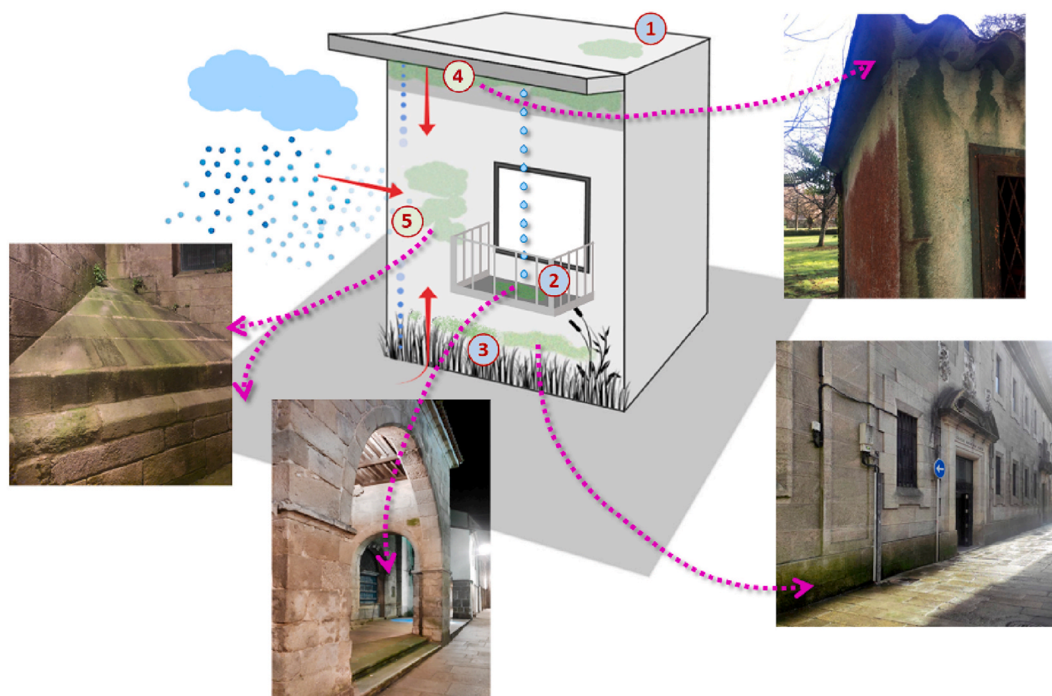

Fig. 1. Potential formation of biofilms following water pathways in a model building. The green circles represent situations simulated by Dynamic methods, and the blue circles represent situations simulated by Static methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

their effects. The use of single species is the simplest approach, as biological interactions are eliminated and understanding the behaviour in response to external changes is of interest. However, the use of multispecies cultures is very important because in nature most biological systems consist of complex consortia rather than monospecific populations, and biofilm communities are assembled as a consequence of interspecies interactions, which dictate community structure, organization and function [25]. Thus, for example, an individual species that is not very resistant to radiation may die when exposed to high levels; however, if the same species develops in a multispecies consortium forming a biofilm, other species can give it protection and increase its survival. Some species may be located in the lower layers, while others develop on the top, giving rise to stratification phenomena (i.e. a layered structure), a typical characteristic of biofilms [11,26].

Table 1 shows the organisms used in different experiments (70 articles). Fig. 2 summarizes the information about the organisms included in Table 1.

In the literature consulted, the use of monospecific cultures predominated (64%) in studies in which commercial methods were used (Fig. 2). Multispecies cultures were used most frequently in studies involving building materials such as stone, tiles, mortar, and cement, in both *Static* and *Dynamic methods* (83 and 62%). Within the multispecies cultures, most were obtained from natural biofilms colonizing built heritage. These biofilms were sampled from building walls and composed of different species, mainly green algae, and cyanobacteria. Portions of the natural biofilms were inoculated into flasks with medium and held under controlled conditions for growth and then inoculated onto sample substrates.

Thus, the most commonly used species in the studies consulted are subaerial epilithic cyanobacteria, together with green algae (Table 1 and Fig. 2). Cyanobacteria are widely distributed and are found in deserts, polar regions, rainforests, and temperate regions, amongst others. They are often associated with water seeps but are tolerant to desiccation. The species most frequently used to develop subaerial biofilms belong the genera *Nostoc*, *Leptolyngbya*, *Chroococcidiopsis*, *Pleurocapsa* and *Aphanocapsa*. Algae, on the other hand, require environments with sufficient water, i.e. climates with more precipitation and mild environmental conditions. In the studies, consulted, the algae most commonly used to produce biofilms in the laboratory belonged to the genera *Chlorella*, *Klebsormidium*, *Trebouxia*, *Apatococcus* and *Coccomyxa*. The diversity of both algae and cyanobacteria depends on water availability [27–29], as this is a limiting factor for photosynthesis. Fungi, often referred to as ecological opportunists, have been reported to a lesser extent in relation to the development of subaerial biofilms on building materials in the laboratory (Fig. 2). However, they are important, as together with heterotrophic bacteria, they are commonly found on weathered rocks [8]. The most frequent genera of fungi used in the studies considered were *Knufia*, *Cladosporium* and *Alternaria*.

From the literature consulted, it was ascertained that two main methods are used to inoculate samples. In all cases, cultures (of monospecies or multispecies) in exponential phase of growth are used. The inoculum of organisms can be pipetted directly onto the samples, which are then incubated under the required conditions of water, temperature and light. Alternatively, the inoculum can be incorporated into a medium and then slowly and continuously applied to the samples. The first method is preferred in *Static methods* and the second in *Dynamic methods*.

3. Laboratory methods for biofilm production

In recent years many studies have tried to address the gap regarding the production of subaerial biofilms by using different methods. These studies have been divided in this review in two main groups -*Static* and *Dynamic methods*-according to the position of samples and the method of supplying water to enhance biofilm development (Fig. 3). The ease of use of each method, as well as the time required, and the similarity of the biofilms produced to natural biofilms will be discussed and the experimental conditions are collected in Table 1. In addition, other methods that cannot be included in these two broad categories will also be described (Sections 3.3 and 3.4).

3.1. Static methods

This group includes methods in which biofilm develops on a horizontal surface on which organisms have been inoculated and water is supplied either by spraying, condensation, or capillary action (Fig. 3A). Water is prevented from flowing over the surface, to prevent the inoculated organisms being washed from the surface. The samples with the inoculated organisms are then incubated under appropriate conditions of humidity and temperature. In *Static methods*, the water supplied can penetrate the substrate from below by capillary action, i.e. samples must be placed in a container with water at a constant level. Alternatively, water can be supplied from above by condensation or by spraying or dripping.

3.1.1. Water supplied by capillary action

In these experiments, samples were placed on a support (trays, Petri dishes, boxes, etc.) filled with water or culture medium up to the surface, without covering the sample. This methodology has been mainly used in bioreceptivity studies of stone and other building materials.

This method was first used and shown to be effective by Ortega-Calvo et al. [30]. In the study, square calcarenite samples were successfully colonized by both a monospecific culture of algae (*Klebsonnidium accidum*) and a monospecific culture of cyanobacteria (*Microcoleus vaginatus*). The samples were held in a climatic chamber for two months at 25 °C with light/dark cycles, and biofilm development was assessed by light microscopy and scanning electron microscope (SEM) techniques. The use of both techniques allowed succession of species to be monitored. The substrates were initially colonized by cyanobacteria, due to their resistance to desiccation as well as their ability to slightly penetrate stone substrates with wide pores, followed by algae colonization. The early colonization by cyanobacteria was observed to facilitate subsequent algal attachment and adhesion, with the subsequent formation of an increasingly complex microbial community. This method was also used with little modifications by other authors. Thus, Tomaselli et al. [31] inoculated horizontally-place marble slabs, pre-saturated with water, with a mixed cyanobacterial culture for 1–2 months at 28 °C; Escadeillas et al. [19], recreated the colonization observed at the base of walls by developing cyanobacterial and green algae biofilms on concrete samples for 3 months at 23 °C; Miller et al. [32] inoculated chlorophyta and cyanobacteria on granite, marble and limestone samples for 4 months at ∼20 °C. Miller et al. [33–35]also developed a non-commercial incubation system including 0.5 cm of sterile water in the bottom of the chamber. A fluorescent lamp was installed at the top of the chamber to provide 12h dark/light cycles, and a constant temperature of 20 ± 2 °C was maintained for 12 weeks. Vázquez-Nion et al. [36] inoculated multispecies cultures on granite samples for 3 months at 23 °C.

Static methods incorporating water by capillary action have also been used to study other porous materials beyond stone. Portillo et al. [21] proposed a standard methodology for studying the bioreceptivity of roofing tiles. These authors tested the susceptibility of five different ceramic roofing tiles to being colonized by the cyanobacteria *Oscillatoria* sp. PCC 9325. The tiles were inoculated and incubated for one month inside a covered glass container with BG11 medium (up to 0.5 cm of the lowest side of the samples). This procedure was also used by Gazulla et al. [37] and Coutinho et al. [38] who added a modification consisting on reinoculation of samples with the same culture every 3 months to simulate the constant deposition of cells that occurs in outdoor environments. Ferrándiz-Mas et al. [39] inoculated with *Chlorella vulgaris* tiles placed in a horizontal position for 21 days at 20 °C. Shirakawa et al. [40] used the same method to induce fungal biofilms for the first time on other materials, such as mortars, by inoculating the upper surfaces with

Table 1
Methods (*Static, Dynamic, Commercial* and *Others*), conditions, substrates and organisms used in laboratory experiments for the successful development of subaerial biofilms. GA: green algae; CY: cyanobacteria; B: Bacteria; F: Fungi; NS: natural biofilm; l/d: light/darkness. Different colours in the substrate column refer to stone (grey), mortars and other materials used in construction (orange), paper and membranes (yellow) and other materials (violet).

STATIC METHODS									
Reference	**Substrate**	**Organisms**		**Method**					**Type of analysis**
				URATION (week	**TEMP (°C)**	**RH (%)**	**LIGHT**	**OTHERS (Water access)**	
Coutinho et al 2016 [38]	Glazed tiles artificially aged	**GA** **CY**	**Green algae:** *Trentepohlia laginefera*, *Chlorella ellipsoidea* and *Apatococcus lobatus.* **Cyanobacteria:** *Nostoc microscopicum*	48	22-23	75-95	Close to a window. No direct sunlight	Water at the bottom	Image analysis Colour measurements Chlorophyll fluorescence SEM
Escadeillas et al 2007 [19]	Concrete	**GA** **CY**	**Green algae**: *Chlorella and Chlorhormidium* **Cyanobacteria**: *Chroococcidiopsis*	12	23	98	1600 lux	Water at the bottom	Image analysis Colour measurements Chlorophyll *a* extraction
Eyssautier-Chuine et al 2015 [20]	Limestone	**GA**	**Green algae**: *Chlorella vulgaris*	4	20	70	PAR 30 mmol photon m^{-2} s^{-1}	Water at the bottom	Colour measurements Chlorophyll fluorescence emission
Ferrándiz-Mas et al 2016 [39]	Tiles of granular waste glass	**GA**	**Green algae**: *Chlorella vulgaris*	3	20±2	-	6500 lux	Water at the bottom	Chlorophyll *a* extraction
Fuentes and Prieto, 2021a [49]	Granite	**GA** **CY**	**Green algae:** *Bracteacoccus minor*, *Chlorella* sp. **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis*	24	23	80	12h l/d cycle	Water at the bottom	Colour measurements Chlorophyll fluorescence CLSM
Fuentes and Prieto, 2021b [6]	Granite	**GA** **CY**	**Green algae:** *Bracteacoccus minor*, *Chlorella* sp. **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis*	6	18/24	80	12h l/d cycle	Water at the bottom (7, 3 and 1 day a week)	Colour measurements Chlorophyll fluorescence
Gazulla et al 2011 [37]	Ceramic roofing tiles	**CY**	**Cyanobacteria**: *Oscillatoria* sp. PCC 9325	2-3	-	-	-	Water at the bottom	PAM fluorometer and colonization height
Genova et al., 2020 [43]	Granite	**GA** **CY**	**Green algae:** *Bracteacoccus minor*, *Chlorella* sp. **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis*	2	23	80	12h l/d cycle	Water at the bottom	Colour measurements
Giannantonio et al 2009a [56]	Concrete	**F**	**Fungus**: *Cladosporium cladosporioides*, *Epicoccum nigrum* and *Alternaria* sp.	2	25	95-100	-	Continuous surface spray cycles	Chlorophyll and carotenoid extraction ESEM
Giannantonio et al 2009b [55]	Concrete	**F**	**Fungus**: *Alternaria* sp., *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Fusarium* sp., *Mucor* sp., *Penicillium oxalicum*, *Pestalotiopsis maculans*, *Trichoderma asperellum*	2	25	95-100	-	Continuous surface spray cycles	Image analysis ESEM
Marques et al 2015 [44]	Schist and granite	**CY**	**Cyanobacteria**: *Nostoc* sp. PCC 9025, *Nostoc* sp. PCC9104 and *Scytonema* sp. CCC9801	17	22	95	12h l/d cycle 1600 lux	Water at the bottom	Colour measurements Chlorophyll extraction
Miller et al 2006 [32]	Limestone, granite and marble	**GA** **CY**	**Green algae**: *Stichococcus bacillaris* **Cyanobacteria**: *Gloeocapsa alpina*	17	20	-	Close to a window. No direct sunlight	Water at the bottom	Area covered Chlorophyll fluorescence emission
Miller et al 2008 [54]	Limestone	**GA** **CY**	**Green algae**: Chlorella, Stichococcus, Trebouxia **Cyanobacteria**: Leptolyngbya and Pleurocapsa.	12	20	-	12 h l/d cycles 1200 lux	Continuous surface spray cycles	DGGE analysis - major microbial components
Miller et al 2009 [48]	Limestone							Water at the bottom	Chlorophyll extraction Chlorophyll fluorescence emission
Miller et al 2010a [33]	Limestone								Chlorophyll extraction Chlorophyll fluorescence emission Image analysis
Miller et al 2010b [34]	Calcarenite								Chlorophyll extraction Image analysis SEM
Miller et al 2011 [35]	Limestone and calcarenite								Chlorophyll fluorescence emission Image analysis
Ortega-Calvo et al 1991 [30]	Calcarenite	**GA** **CY**	**Green algae**: *Klebsormidium flaccidum* **Cyanobacteria**: *Microcoleus vaginatus*	8	25	-	light/dark cycle 250 lux	Surface condensation	Microscopic observations SEM
Papida et al 2000 [22]	Dolomite and limestone	**NS**	Mixed microbial populations	3	27	-	-	Continuous surface spray cycles	Bacterial cell counts Total carbohydrate SEM
Portillo et al 2011 [21]	Five different ceramic roofing tiles	**CY**	**Cyanobacteria**: *Oscillatoria* sp. PCC 9325	4	-	-	-	Water at the bottom	2D scanning of the surface Chlorophyll fluorescence emission Microphotographs under white light illumination and autofluorescence
Prieto and Silva 2005 [57]	Granite	**CY**	**Cyanobacteria**: *Nostoc* sp. PCC 9025, *Nostoc* sp. PCC9104 and *Scytonema* sp. CCC9801	9	25	95	12 h l/d 800 lux	Surface condensation	Chlorophyll extraction
Prieto et al 2006 [51]	Quartz from a quarry	**CY** **B**	Culture containing organisms isolated directly from the mine,mainly **cyanobacteria** together with **bacteria**	14	20	100	12 h l/d 600 lux	Surface condensation	Colour measurements
Prieto et al 2014 [58]	Granite treated with plastic-based products	**GA** **CY**	**Green algae** **Cyanobacteria** (Not identified; from natural green biofilms)	6	26	75	12 h l/d	Surface condensation	Colour measurements Chlorophyll extraction
Prieto et al 2020 [50]	Granite	**GA** **CY**	**Green algae**: *Chlorella* sp. and *Stichococcus bacillaris* **Cyanobacteria**: *Aphanocapsa fuscolutea*, *Gloeocapsa aeruginosa*, *Gloeocapsa punctata* and *Isocystis* sp	9	25	100	12 h l/d 1900 lux	Water at the bottom	Colour measurements Chlorophyll extraction EPS quantification
Ramil et al 2020 [59]	Granite	**GA** **CY**	**Bryophyta**: *Syntrichia ruralis* **Green algae:** *Klebsormidium* sp., *Bracteacoccus* sp., *Chlamydomonas* sp., *Chlorella* sp. and *Stichococcus bacillaris* **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis*	3	23	95	12 h l/d 31 μmol photons m^{-2} s^{-1}	Surface condensation	Hyperspectral Imaging
Sanmartín et al 2019 [23]	Schist	**GA** **CY**	**Green algae**: *Klebsormidium* sp., *Bracteacoccus* sp., *Chlamydomonas* sp., *Chlorella* sp. and *Stichococcus bacillaris* **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis*	6	23-27	95	12 h l/d 25 μmol photons m^{-2} s^{-1}	Water at the bottom	Colour measurements Chlorophyll fluorescence
Sanmartín et al 2020 [47]	Granite	**NS**	Natural biofilm	7	28±1	90	16:8 h l/d	Water at the bottom	Colour measurements Chlorophyll fluorescence Surface roughness - optical profilometer
Sanmartín et al 2021 [24]	Granite	**GA** **CY**	**Green algae:** *Bracteacoccus minor* **Cyanobacteria:** *Nostoc sp. PCC9104*	11	20.3	90	9.55 photons m^{-2} s^{-1}	Surface condensation	Chlorophyll fluorescence Colour measurements Image analysis Pigment quantification SEM.
Sasso et al 2016 [42]	Limestone with biocidal treatments	**GA** **CY**	**Green algae**: *Chlorella*, *Stichococcus*, and *Trebouxia* **Cyanobacteria**: *Leptolyngbya* and *Pleurocapsa*	3	20±2	-	Close to a window. No direct sunlight	Water at the bottom	Colour measurements Image analysis Pigment quantification CLSM.
Shirakawa et al 2003 [40]	Mortars	**F**	**Fungi**: *Cladosporium sphaerospermum*	4	25	-	-	Water at the bottom	Stereomicroscope SEM
Tomaselli et al 2002 [31]	Marble treated with biocides	**CY**	**Cyanobacteria**: *Synechococcus* ML1, *Gloeocapsa* ML3, *Gloeocapsa* ML6, *Coccomyxa* ML18, *Leptolyngbya* ML8, *Leptolyngbya* ML9, *Nostoc* ML15 and *Coccomyxa* ML19	4-8	28	-	16:8 h l/d	Water at the bottom	Chlorophyll fluorescence
Trovão et al., 2020 [52]	Limestone	**F**	Natural isolated fungi at the Old Cathedral of Coimbra.	52	Room t[a]	-	-	Every three months water + glucose was added to the surface	Light microscopy SEM Micro-Raman Spectroscopy
Trovão et al., 2021 [53]	Limestone	**F**	*Aeminium ludgeri , Aspergillus glaucus , Aspergillus westerdijkiae , Penicillium brevicompactum and Penicillium chrysogenum . Aspergilli and Penicillia*						
Urzì and De Leo 2007 [41]	Mortars with water repellents and biocides	**F** **B** **GA** **CY**	**Fungi**: *Alternaria alternata* and *Cladosporium* sp., *Humicola*, *Ulocladium*, *Phoma* **Bacteria**: *Micrococcus* sp. **Cyanobacteria** *Nostoc* sp. **Green algae** *Chlorella* sp.	156	Env.T[a]	-	Natural light	Water at the bottom	Light and epifluorescent microscopic analysis

Reference	Substrate		Organisms	DURATION (weeks)	TEMP (ºC)	RH (%)	LIGHT	OTHER (Water access)	Type of analysis
Vázquez-Nion et al 2016 [36]	Granite	GA CY	**Green algae**: *Chlorella* sp. and *Stichococcus bacillaris*) **Cyanobacteria**: *Aphanocapsa fuscolutea*, *Gloeocapsa aeruginosa*, *Gloeocapsa punctata* and *Isocystis* sp.	8	23	95	12 h l/d 20 µmol photons m^{-2} s^{-1})	Water at the bottom	Colour measurements CLSM EPS quantification
Vázquez-Nion et al 2018a [45], 2018b [46]	Granite	GA CY	**Green algae**: *Klebsormidium* sp., *Bracteacoccus* sp., *Chlamydomonas* sp., *Chlorella* sp. and *Stichococcus bacillaris.* **Cyanobacteria**: *Aphanocapsa* sp. and *Leptolyngbya cebennensis.*	12	23	95	12 h l/d 20 µmol photons m^{-2} s^{-1}	Water at the bottom	Chlorophyll fluorescence Colour measurements EPS quantification

DYNAMIC METHODS

Reference	Substrate		Organisms	Method					Type of analysis
				DURATION (weeks)	TEMP (ºC)	RH (%)	LIGHT	OTHER (Water access)	
Barberousse et al 2007 [60]	Manufactured mortars, organic finish (ETICS) and paint	GA CY	**Green alage**: *Klebsormidium flaccidum*, *Chlorella mirabilis* and *Stichococcus bacillaris.* **Cyanobacteria**: *Chroococcidiopsis fissurarum*	4 – 7	24	50±5	25±5 mmol $s^{-1}m^{-2}$PPF	45° inclination	Image analysis
Barriuso et al 2017 [76]	Calcareous stones + consolidation treatment with biocides.	GA CY	**Green algae**: *Tribonemales*, *Chlorellales* **Cyanobacteria**: *Nostocales*, *Oscillatoriales* and *Chroococcales*).	8	26–28	-	12 h l/d 50 µmol $s^{-1}m^{-2}$	BG11 media sprinkled 1 h/day 30° inclination	Colour measurements Macro images Optical microscope Chlorophyll fluorescence.
De Muynck et al 2009 [67]	Concrete with water repellents and/or biocides	GA	**Green algae**: *Chlorella vulgaris* var. *viridis Chodat*	12	19.5-21.5 (n/d)	86–93 (d/n)	12 h l/d 800 lux	45° inclination.	Colour measurements Image analysis
D'Orazio et al 2014 [72]	ETICS and clay bricks	GA CY	**Green algae**: *Chlorella mirabilis* **Cyanobacteria**: *Chroococcidiopsis fissurarum*	60	24	-	14:10 h l/d 1500 lux	45° inclination.	Image analysis Colour measurements CLSM
Dubosc et al 2001 [62]	Mortars	GA	**Green algae**	8	21-25	80-95	2000 lx	Int. sprinkling 45° inclination	Covered area
Escadeillas et al 2007 [19]	Concrete	GA CY	**Green algae**: *Chlorella and Chlorhormidium* **Cyanobacteria**: *Chroococcidiopsis*	8-12	21	98	1600 lux	Culture sprinkled 3 h/day 45° inclination	Image analysis Colour measurements Chlorophyll *a* extraction
Goffredo et al 2017 [74]	TiO2-based nano-compounds applied on travertine	GA	**Green algae**: *Chlorella* sp., *Klebsormidium* sp., *Phormidium* sp. and *Chlorogloeopsis* sp.	9	25 ± 3.3	97.7 ± 2.8	12 h l/d	45° inclination.	Colour measurements Image analysis
Graziani et al 2013 [68], 2014 [69], 2016a [70], Graziani and D'Orazio 2015 [73]	Clay bricks with TiO2 nano-coatings	GA	**Green algae**: *Chlorella mirabilis* and Cyanobacteria: *Chroococcidiopsis fissurarum*	9	24	80-90	14:10 h l/ d	Suspension sprinkled 15 min for 6 h 45° inclination	Colour measurements Image analysis
Graziani et al 2016b [71]	Fired bricks with TiO2-Cu and TiO2-Ag nano-coatings	GA CY	**Green algae**: *Chlorella mirabilis* **Cyanobacteria**: *Chroococcidiopsis fissurarum*	7	-	50-100	14:10 h l/d	45° inclination	Colour measurements Image analysis
Graziani and Quagliarini 2018 [75]	Limestone and sandstone coated with Nano-TiO2	GA CY	**Green algae**: *Chlorella mirabilis* **Cyanobacteria**: *Chroococcidiopsis fissurarum*	8-10	24	80-90	1500 lux	45° inclination	Image Analysis
Guillitte and Dreesen 1995 [61]	Building materials mortars and concrete	GA CY F	**Green alagae** **Cyanobacteria** **Fungi**	24-36	25-30	80-90	16:8 h l/d	Certain inclination	Image analysis Microscopy
Manso et al 2014 [66]	Magnesium phosphate cement (MPC)	GA	**Green algae**: *Chlorella vulgaris*	12	25-22 d/n	82-99 d/n	12 h l/d	45° inclination	Colour measurements Image analysis Chlorophyll *a* fluorescence emission
Quagliarini et al 2019 [29]	Clay bricks	GA CY	**Green algae**: *Chlorella mirabilis* **Cyanobacteria**: *Chroococcidiopsis fissurarum*	8	27.5 ± 2.5 10 ± 2.5	75 87 98	14:10h l/d 1500 lux	45° inclination	Colour measurements Image analysis EPS quantification
Tran et al 2012 [63], 2013 [64], 2014 [65]	Mortars	GA	**Green algae** *Klebsormidium flaccidum*	12	24	-	12 h/day	45° inclination Sprinkling 90 min/12 h	Colour measurements Image analysis

OTHER METHODS

Reference	Substrate		Organisms	Method					Type of analysis
				DURATION (weeks)	TEMP (ºC)	RH (%)	LIGHT	OTHERS (Water access)	
Bertuzzi et al 2017 [83]	Sterile filter paper discs	GA	**Green algae**: *Coccomyxa subellipsoidea*, *Coenochloris* sp., *Pleurochloris* sp., *Stichococcus bacillaris*, *Apatococcus lobatus* and *Trebouxia gelatinosa*	4	20 ± 1	-	14:10 h l/d 20 ±1 mmol photons m-2 s-1	Water obtained through agar plates	Chlorophyll fluorescence Epifluorescence Quantification of low molecular weight carbohydrates (not EPS)
Blanken et al 2014 [87]	Stainless steel and polycarbonate	GA	**Green algae**: *Chlorella sorokiniana*	1	38	-	Warm white 45 mil chip	Rotation of disks 42% submerged in medium	Biomass
Breitenbach et al 2018 [82]	Cellulose acetate filters	F	**Fungus** *Knufia petricola*	1	25	-	-	Water obtained through agar plates	EPS quantification
Favero-Longo et al 2009 [85]	One limestone and four white marbles		Mycobionts and photobionts isolated from specimens of *Bagliettoa baldensis* and *Bagliettoa marmorea* (lichens)	12	15	-	-	Water obtained through agar plates	Reflected light stereomicroscope.
Gambino et al 2019 [79]	Polycarbonate filter membranes	CY	**Cyanobacteria**: *Nostoc* sp. PCC 9104	4	23.3-22.5	-	12 h l/d	Water obtained through agar plates	Biomass and protein content Colour measurements Pigment quantification EPS quantification ATP Reactive oxygen species Microscopy
Huang et al 2016 [84]	Microfiltration membrane	GA	Green algae: *Chlorella vulgaris*	1	25	-	-	Water obtained through agar plates	Dry biomass weight
Marasco et al 2016 [86]	*Tesserae* mainly composed by calcite	GA CY	**Green algae**: *Chlorella vulgaris*, *Coccomyxa solorinae-saccatae*, *Coelastrella rubescens*, *Pseudococcomyxa simplex*, *Scenedesmus communis* and *Stichococcus bacillaris.* **Cyanobacteria**: *Aulosira terrestre*, *Calothrix membranacea*, *Fischerella ambigua*, *Microchaete diplosiphon*, *Microcoleus autumnalis*, *Nodularia sphaerocarpa*, *Nostoc commune*, *Plectonema*sp. and *Scytonema mirabile*	6	20 ± 2	98-100	16:8 h l/d 150 µmol photon m-2 s-1	Subaquatic + water at the bottom	Image analysis CLSM
Sanmartín et al 2011 [77]	Polycarbonate filter membranes	CY	Cyanobacteria: *Nostoc* sp. PCC 9104	4	22-23		daylight with a 12-hlight/12-h-dark period.	Water obtained through agar plates	Colour measurements Chlorophyll quantification ATP measurement Microscopic observations

COMMERCIAL METHODS

Reference	Substrate		Organisms	Method					Type of analysis
				DURATION (weeks)	TEMP (ºC)	RH (%)	LIGHT	OTHERS (Water access)	
Vázquez-Nion et al 2020 [90]	Granite	CY	**Cyanobacteria**: *Synechocystis* sp. PCC 6803	3	Room tª	-	14:10 h l/d 30 µmol photon m-2 s-1	Continuous flow	Cell counts Pigment quantification EPS quantification Oxidative stress CLSM
Villa et al 2015 [26]	Limestone	CY B	**Cyanobacteria**: *Synechocystis* sp. PCC 6803 **Bacteria**: *Escherichia coli* K12	2	Room tª	-	14:10 h l/d 40 µmol photon m-2 s-1	Continuous flow	Cell counts SEM EPS quantification Oxidative stress CLSM Cryosectioning and measuremeny of biofilm thickness Oxygen and pH microsensor measurements
Gerrits et al 2021 [88]	Polished olivine sections	F	**Fungus**: *Knufia petricola*	28	25	-	Continuous light 90 µmol photons m−2 s−1).	Flow rate 0.030 ml min−1	SEM and cryo-SEM FIB-TEM

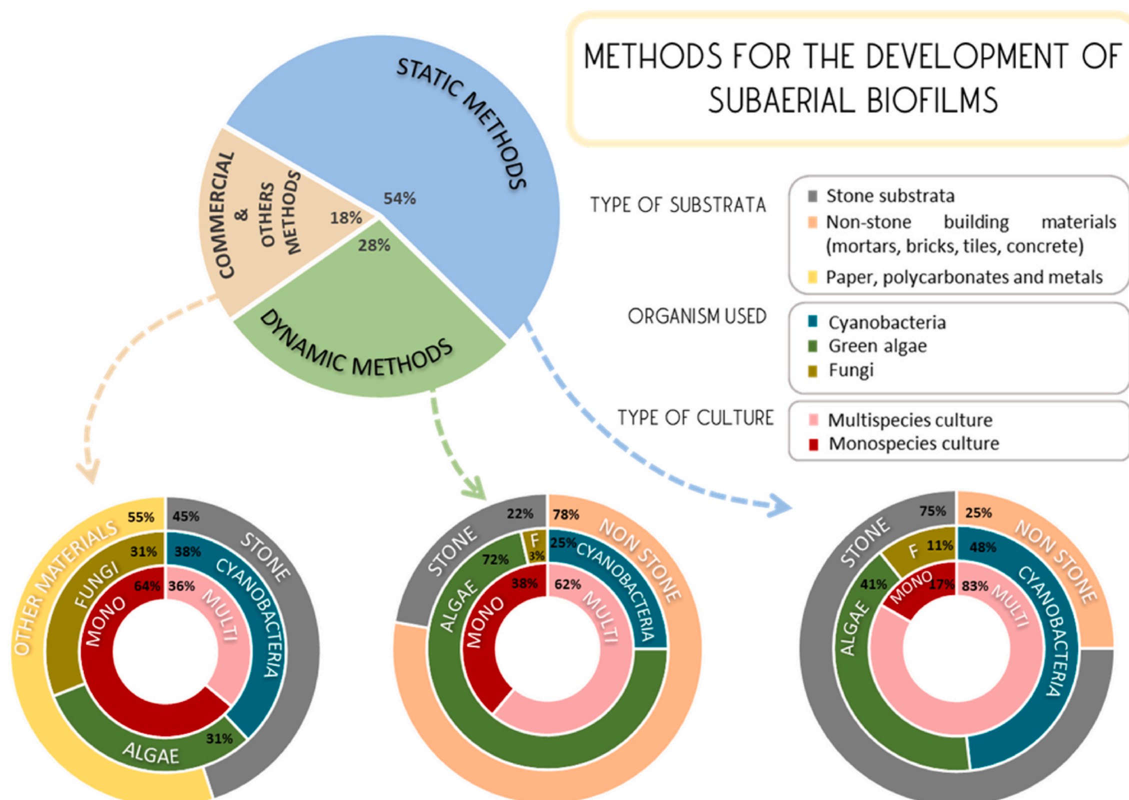

Fig. 2. Proportion of scientific publications reporting the use of substrate (stone, non-petroleum materials such as mortar, tiles, brick or cement and other materials such as paper, polycarbonate and metal), type of organism (cyanobacteria, green algae and fungus) and type of culture (multispecies and monospecies). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a spore suspension, spraying the surfaces with nutrient medium with water and incubating the samples for one month at 25 °C.

These methodologies have also been used in studies aimed at understanding the effects of different treatments (biocides, consolidants or paints) applied to stone [20,23,24,41–43], the type of substrate, degree of weathering or colour [44–48] and the influence of environmental parameters [6,49,50] on the development of organisms.

3.1.2. Spraying and condensation

In other cases, the water was supplied by deposition on the surface of the samples, rather than by capillary action. Thus, the water was supplied by **spraying or dripping** or by inducing **condensation**. Researches using **spray supply** are the following. Prieto et al. [51] compared the effect on biofilm development of spraying medium or water on to quartz samples inoculated with cyanobacteria. After 100 days, biofilm development was found to be highest in the samples sprayed with medium. Trovão et al. [52,53] also used fungi to test the bioreceptivity of limestone samples. The surfaces of the limestone samples were inoculated with fungi, and the samples were maintained at room temperature for 12 months, with a three-monthly addition of water or water with glucose on the surface to recreate natural conditions. Papida et al. [22], developed a natural biofilm on dolomite and limestone, using a natural biofilm and reducing the experiment duration to 21 days at 27 °C.

Miller et al. [54] developed a non-commercial chamber in which six replicate stone samples were placed horizontally for inoculation of materials using an automatic irrigation system with a preestablished periodicity. During the first two weeks of the study, the stone samples inside the chamber were inoculated with the phototrophic culture, which passed through a sprinkler system and was projected on the top of the stone samples for 5 min every 8 h. After each watering session, the liquid deposited in the chamber was collected, recycled by a pump and redirected to the sprinklers. The stone samples were sprinkled with 1000 × diluted BG11 medium for 5 min every 8 h for a period of two weeks. The samples were then sprinkled with water for 5 min every 12 h, to simulate outdoor environmental conditions. This method was also used by Giannantonio et al. [55,56], with fungi instead of algae.

In some studies, biofilm development was induced by creating environmental conditions favouring water **condensation** on the sample surface [57–59]. Prieto and Silva [57] successfully induced biofilms on granite blocks by inoculating the blocks with a mixed cyanobacterial culture and incubating them for two months at 25 °C in a climatic chamber with constant and light/darkness cycles and high relative humidity (95%) as only water source. Ramil et al. [59] used the same procedure to produce biofilms from a multispecies culture composed of green algae and cyanobacteria, and maintaining it for 21 days at 23 °C, with a photoperiod of 12/12 h light/darkness and 95% relative humidity as the water source. Biofilms were even developed when samples were held at a lower relative humidity (75%), as in studies on biocidal effect of plastic-based products applied to granite Prieto et al. [58].

These different *Static* water supply methods (spray, condensation, and capillarity) are recommended for recreating biofilms found naturally on horizontal surfaces, such as balconies or pavements, where the substrate surface is intermittently covered by a layer of water and the biofilms are exposed to air and also to water. The main limitations of *Static methods* seem to be in achieving homogenous cover of the surfaces and good adhesion to the substrate, as well as maintaining constant humidity levels (or water availability) necessary for the correct development of the biofilm.

3.2. Dynamic methods

Dynamic methods include simulation of semi-continuously circulation of rainwater over the substrate to promote strongly anchored biofilms.

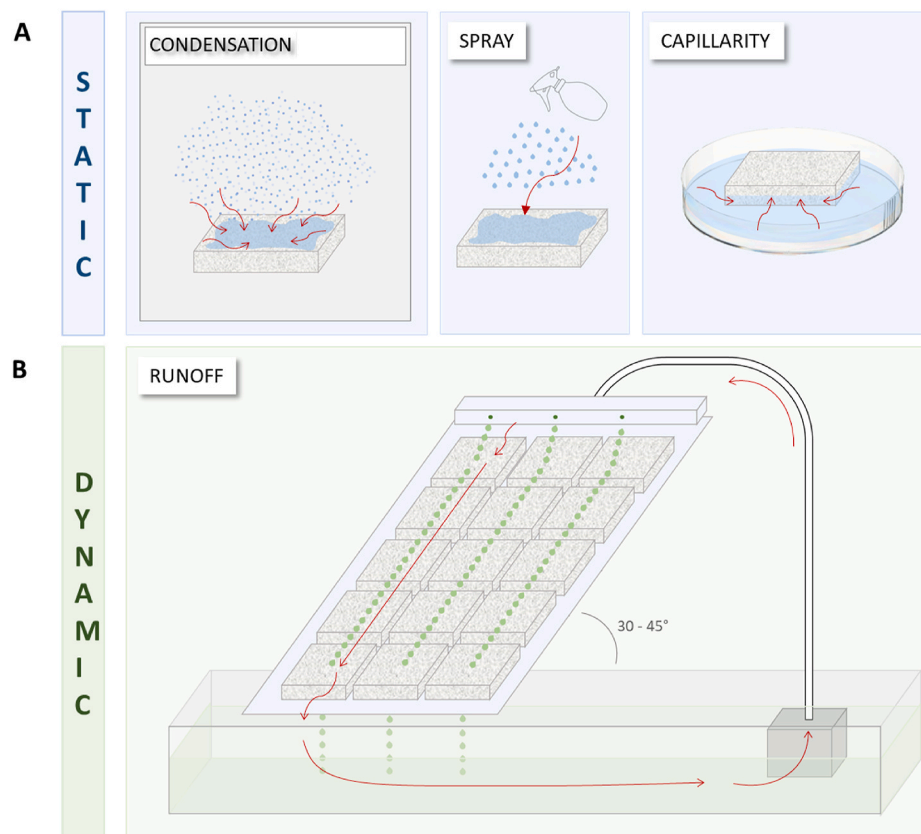

Fig. 3. Diagram of the set-ups for Static (blue; where water is supplied by condensation, spray, or capillary action) and Dynamic (green, where water flows intermittently over the substrate surface) methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In these methods, the organisms growing on the substrate must overcome the drag forces of the current and they will thus be well anchored. The main characteristic of the various *Dynamic methods* is the position of the samples, always inclined between 30° and 45° (Fig. 3B). The advantage of inclining the substrate samples is the increased time during which water flowed over the surfaces [60].

Guillitte and Dreesen [61] developed a system to test the bioreceptivity of different building materials (including stone, bricks, mortar and concrete), as assessed by biofilm induction. Sample blocks were placed in water-collecting containers with 80–90% of relative humidity, temperature ranging between 25 and 30 °C and 16h of lighting per day. Samples of different materials were placed on rests at a certain angle, after being inoculated with a mixed culture (cyanobacteria, algae and mosses). Throughout the 6–9 months of the experiment, the blocks were wetted by a water sprinkler system in which the water was projected on the top of the blocks. Adjustment of the inclination of the samples allowed water to run over the surface of the samples. The water used was enriched with diaspores of cyanobacteria, algae and mosses. During the first 6 months of the experiment, the water was projected, through the sprinklers, on to the top of the rests for 15 min every 2 h. During the last 3 months of the experiment, the wetting period was reduced to once every 8 h. The water was recycled in the system after being collected by a device consisting of collecting reservoirs and a pump. Similarly, Dubosc et al. [62], Barberousse et al. [60] and Escadeillas et al. [19] used a water-streaming test that mimics the conditions under which facade materials are colonized, by producing a stream of cultured microorganisms or water on top of materials. adapting it to the needs of each study. Dubosc et al. [62] placed mortar samples (not previously inoculated) at an angle of 45° and an algal and cyanophyceae culture with water and medium was run over them for 3 h/day. The system was maintained at 21-25 °C, and a biofilm developed after two months. Barberousse et al. [60] produced a multispecies chlorophyte and cyanobacterial biofilm on mortar samples held at 23 °C for 30–50 days. The chamber was filled with 50 L of BBM enriched medium with algae or cyanobacteria cultures. The sprinkling cycles were set to start every 12 h and to run for 90 min over the samples inclined at an angle of 45°. Escadeillas et al. [19] also use this system on mortar plates inclined at an angle of 45° in a chamber where water containing a culture of green algae and cyanobacteria was allowed to run off. Total coverage was achieved in 2 months at 3 h/day run-off conditions and 3 months at 1 h/day run-off conditions and 21 °C. On the basis of the research reported by Barberousse et al. [60], Tran et al. [63–65] designed a closed glass chamber (100 x 50 × 50 cm) with 50 l of an algae suspension maintained at 24 °C by means of a thermoregulator in order to study the susceptibility of different types of mortar to being colonized. In this device, two rows of mortar samples were placed back-to-back on a stainless-steel support inclined at 45°; this allowed the suspension of algae to flow on the upper surface of each sample. Light was provided by neon lamps and the samples were held in the device for 3 months. Manso et al. [66] also tested the bioreceptivity of cementitious materials in a study designed to stimulate biological growth. The accelerated algal fouling test was conducted in a modular water run-off test developed by De Muynck et al. [67]. The set-up consisted of 6 stainless steel compartments inclined at an angle of 45°. The run-off period was set to start every 12 h and ran for 90 min and a 12 h day and night regime was followed. Temperature and relative humidity ranged between 22 °C (night)–25 °C (day) and 82% (day)–90% (night).

Dynamic methods have also been used in more complex studies examining e.g. the effect of climatic conditions on colonising biofilms or the effects of different biocidal and consolidating treatments. In this respect, Quagliarini et al. [29] first used this dynamic system (samples inclined at 45°, incubation for 8 weeks), in combination with the

application of different climatic parameters (27 and 10 °C and 75%, 87% and 98% of relative humidity). These authors showed that at temperatures higher than 35 °C, the presence of the green alga *Chlorella mirabilis* decreased significantly, while growth of the cyanobacterium varied regardless of the environmental temperature. They also found that RH < 98% could be assumed to protect against algae growth on fired bricks, independently of substrate properties. The dynamic system or rain simulator has also been used successfully to study the effect of different treatments on the development of organisms on both stone substrates and other materials with a clay component, such as mortar, cement and bricks. De Muynck et al. [67] used a dynamic system to produce a biofilm composed by *Chlorella vulgaris*. The authors tested the anti-biofouling characteristics of different products applied to concrete in the system, held for 3 months at 19.5–21.5 °C. Graziani et al. [68] [–] [71], D'Orazio et al. [72], Graziani and D'Orazio [73] and used the system to test the inhibitory effect of TiO2 nanocoatings on the development of biofilms composed by *Chlorella mirabilis* (Chlorophyta) and *Chroococcidiopsis fissurarum* (Cyanobacteria) on clay bricks.. Goffredo et al. [74] produced biofilms on travertine rock covered by a TiO2-based nano-coating. The samples were placed in a tank was filled with medium with algae, which was inoculated directly on stone surfaces through drip irrigation, whereby the medium flowed over the sample surfaces. Graziani and Quagliarini [75] tested the bioreceptivity of both sandstone and limestone, which were coated using the methodology described by Graziani et al. [68]. Barriuso et al. [76] induced formation of biofilms composed of different algae and cyanobacteria to test the effectiveness of a consolidation treatment preventing colonization on calcareous stone. For this purpose, an accelerated growing chamber, consisting of a glass tank filled with the inoculum solution in which the samples were inclined above the water level at an angle of 30°, was used. The inoculum solution was gently sprinkled over the sample for 1 h/day, in a closed loop system. A continuous aeration system was placed on the bottom to move and oxygenize the inoculum solution. Biofilm was successfully developed in the system, which was run for 2 months at 26–28° with 12 h daylight lamp cycles (50 μmol/s·m2).

These *Dynamic methods* are recommended for recreating the type of biofilms found naturally on vertical and inclined surfaces on which water runs, such as walls and roofs. The main limitation of *Dynamic methods* seems to be the time required to achieve a thick biofilm relative to other methods. However, these methods have the advantage that they allow the development of strongly attached biofilms because of the drag forces they undergo.

3.3. Other laboratory methods

In addition to the above-mentioned methods, some other methods have been developed specifically for the study objectives. The techniques described below are not used in built heritage studies, but they may be useful for future use in this field of research. Due to their novelty and ease of use, we believe that they should be described as potentially useful methods for the study of the development of biofilms on building materials.

The use of agar culture plates enriched with different nutrients enables study of individual organisms, since specific culture media will accelerate the development of specific organisms. The use of medium enriched agarized plates proved useful for producing biofilms on polycarbonate membranes (Fig. 4; A), in order to study biofilm composition, biofilm architecture and organism physiology, where the effect of the substrate on biofilm formation was not the object of the study. In these cases, biofilms were produced on membrane filters resting on enriched agar plates. Water and nutrients were passed through the permeable membrane and thus reach the organisms (Fig. 4; A). The main advantage of this method is that it allows the use of study techniques such as CLSM, optical microscopy and RAMAN, which would be more difficult to apply when using solid, opaque and heavy materials such as metal, stone, concrete or mortar. Sanmartín et al. [77] used the mechanism previously developed by Ref. [78] to induce *Nostoc* sp. *PCC 9104* biofilms on polycarbonate membrane filters, to study whether colour measurements could be used as an indicator of chlorophyll degradation and to develop noninvasive and non-destructive study methodologies for cultural heritage studies. Polycarbonate filter membranes resting on agar plates were inoculated and cultured for 30 days (transferred to fresh agar plates every 3 days) in a controlled environment under simulated daylight with a 12-h light/12-h dark period. The temperature inside the cabinet was 22–23 °C. This method was also used by Gambino et al. [79] to study the effect of surface colour on the development of cyanobacteria and also by Sanmartin et al. [80] to test the effect of UV-A or UV-B radiation plus red LED light on the formation of biofilms commonly found on buildings. In addition to polycarbonate membranes, other authors have used paper filters, which have been shown to be useful for producing fungal biofilms, in addition to phototrophic biofilms. On the basis of the research reported by Gorbushina and Broughton [81], Breitenbach et al. [82] produced biofilms of the rock-inhabiting fungus *Knufia petricola* on cellulose acetate filters placed on malt-extract agar plates (MEA) by spraying a cell suspension of the fungus and incubating the samples at 25 °C for 7 days. Bertuzzi et al. [83] also used filter paper with the aim of studying the applicability of heat shock treatments to six morphologically and phylogenetically distant green microalgae. The microalgae cultures were grown at 20 ± 1 °C, with a light/darkness regime of 14/10 h, and 20 ± 1 mmol photons m-2 s-1 for 4 weeks before exposure to treatments. This same principle was used by Huang et al. [84] to design a bioreactor for biofilms development on microfiltration

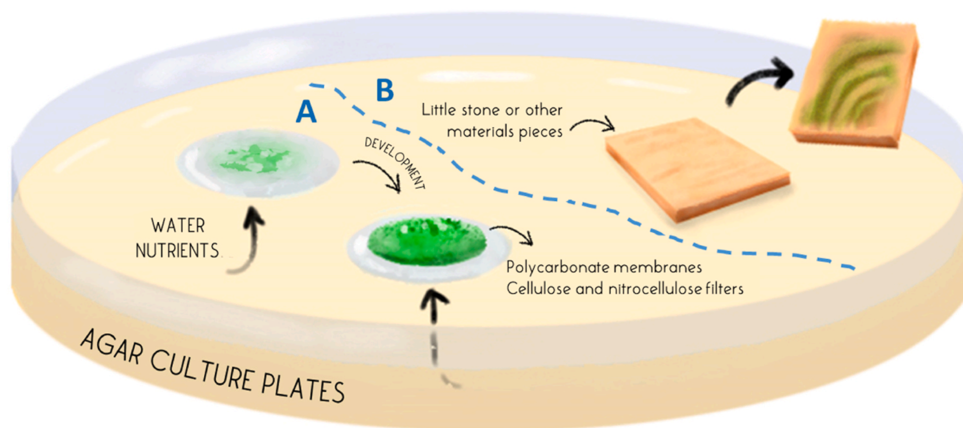

Fig. 4. Diagram of the set-up for the development of biofilms using agar culture plates with membranes in which the biofilm grows in contact with air (A) or with pieces of rock or other materials, growing the biofilm in contact with agar (B).

membranes. In this case, *Chlorella vulgaris* was used as the test organism. Bioreactors consisting of boxes (300 x 60 × 50 mm) made of polymethyl methacrylate, with fluorescent lamps and a pipe aerator for CO2 sparging, were used to grow microalgae biofilms. Agar medium containing BG11 was poured into bioreactors to provide nutrients and maintain the wettability of the algal biofilm. A filtration membrane (diameter, 50 mm, pore size, 0.45 lm) was inoculated by vacuum filtration and attached to the surface of the solidified BG11 medium to serve as a point of adherence for microalgal biofilm growth.

Exceptionally, agar culture plates have been used to develop biofilms previously inoculated on lithic surfaces [85] (Fig. 4; B). Limestone and marble samples were inoculated and incubated (13–15 °C, with light) on plates with agar media, on which mycobiont and photobiont colonies mainly developed on the face of the samples in contact with the agar (Fig. 4; B). After incubation of the samples for one year, mycobionts had developed on the surface and inside all lithotypes, and different penetration pathways that depended on mineralogical and structural characteristics and different receptivity were observed. By contrast, inoculated algae did not penetrate the samples.

Other methods used to develop biofilms that are not strictly subaerial are mentioned below. These methods are considered here because of their potential use in studying the biodeterioration of built structures. Marasco et al. [86] studied the bioreceptivity of tesserae from the mosaics in Roman Thermal Baths of via Terracina (Naples, Italy) by immersing them, previously inoculated, in BBM medium, placed horizontally in flasks and incubated for 1 month. The tesserae were then aseptically collected from the flasks and transferred to sealed glass chambers and incubated for another 2 weeks under the same conditions of light, temperature (20 ± 2 °C) and humidity (98 and 100%). Likewise, Blanken et al. [87] developed an innovative device, the Rotating Biological Contactor Based Photobioreactor (Algadisk photobioreactors), to produce biofilms from green alga *Chlorella sorokinian*. The device consisted of a container, four discs (two stainless steel discs of different thicknesses and one sanded polycarbonate disc were tested) and eight lamps. The discs, installed in a rotor, were immersed 42% of their surface in inoculated medium. The temperature was maintained at 38 °C and the discs were rotated with only the tops illuminated. This approach has the advantage of allowing selective pressure to stimulate biofilm growth while minimizing growth of microalgae in the suspension. The idea behind this mechanism is to produce algal biomass, and under optimal conditions 20.1 ± 0.7 g of biomass was produced per m2 of disc surface per day.

3.4. Commercial biofilm reactors and industrial test standards

Some commercially designed systems are available for biofilm formation and can be used to study biofilms sampled from built cultural heritage. The Drip Flow Biofilm Reactor® (DFR) consists of four or six parallel test channels, each capable of holding one standard glass microscope slide sized sample. In this device, medium is supplied by dripping over the surface of the sample or catheter. The CDC Biofilm Reactor® consists of eight polypropylene sample holder rods suspended from a polyethylene ported lid. The rods can each accommodate three sample discs of diameter 1/2 in. (12.7 mm). The lid with samples holders and sample discs is mounted in a glass vessel (1l) with side-arm discharge port. The liquid growth media is circulated through the vessel while mixing, and shear is generated by a magnetic stirring bar. Both systems (and some variations developed by the same company) have been successfully used by different authors to produce biofilms (e.g. Ref. [88]), especially bacterial biofilms on slides and coverslips [89], and also to recreate the biofilms typically found on buildings [26,90]. These technologies have the advantage of allowing exact replication of the experiments but have the disadvantage of being difficult to transfer to hard materials due to the size and shape of samples, as well as the difficulty associated with cutting some materials.

Moreover, many standard test methods have been developed by standardisation agencies and organisations. These tests have mainly been developed for studying biodeterioration processes and biocidal efficacy and involve the use of both algae (e.g. SS345; ASTM G 29–10) and fungi (e.g. EN 60068-2-10; WK4201; ISO 16869:2008; ASTM D 2020–03) on different materials. Many of the existing regulations, in addition to the examples given above, are included in the ECHA's Guidance on the Biocidal Products Regulation [91]. Although these methods have not been standardised for building materials, they could be considered in future studies.

4. Concluding remarks

With the aim of providing a guide to selecting the most appropriate method for cultivating SABs on different building materials, a total of 70 published papers were consulted. All of the methods reviewed resulted in the successful development of biofilms, and there is therefore a great deal of scope in terms of the conditions that can be used. The type of biofilm to be replicated and the time and equipment available will determine the choice of one method or another.

Building architecture demarcates water pathways, and water availability is known to be the main factor determining biofilm development. Thus, water is available to biofilms colonizing different building structures in different ways. Thus, in laboratory experiments, the choice between *Static* or *Dynamic methods* must be made considering the availability of water to the biofilm to be replicated. *Dynamic methods* will provide more information when simulation of the washing effect of rainwater is the most important factor, while *Static methods* will be more appropriate for studying colonization of areas where the water does not exert a dragging force. Thus, *Static methods* will be more appropriate for studying e.g. the bioreceptivity of materials used in balconies or other horizontal structures. However, to study the effectiveness of the biocidal effect on colonized walls exposed to rainfall, *Dynamic methods* will be more appropriate. Moreover, both methods have some limitations that must also be considered: *Static methods* are faster than *Dynamic methods*, but the latter result in more strongly attached biofilms than the former.

In addition to *Static* and *Dynamic methods*, other methods involving e.g. agar plates or bioreactors can be used complementarily when the technical limitations of the monitoring device do not enable analysis of hard substrates such as stone, mortars and tiles. Use of such methods also has the advantage of reducing the time required for biofilm formation and increasing the replicability.

The microorganisms mainly used to develop biofilms are algae and cyanobacteria and to a lesser extent fungi, irrespective of the method used. Mixed cultures developed from natural biofilms were mainly used, although in some studies monospecies cultures of model or ubiquitous species have been also employed. The choice of mixed cultures or monospecies cultures depends on the final purpose of the study. Thus, in case studies it is important to develop biofilms that are as similar as possible to those colonising the study site. In that case, mixed cultures typical of natural environments should be used. Nevertheless, if the physiological or metabolic aspects of biofilms are particularly important, use of model monospecies cultures is recommended.

Regarding the experimental conditions, the most common conditions resulting in successful formation of mature biofilms in the studies reviewed were a temperature of between 20 and 24 °C, relative humidity of more than 90% and incubation for 12 weeks.

The above considerations will be of great help to homogenize methods of developing biofilms and will thus facilitate comparison between studies.

CRediT authorship contribution statement

Elsa Fuentes: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Daniel Vázquez-Nion:** Data curation, Conceptualization. **Beatriz Prieto:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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