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The Porifera microeukaryome: Addressing the neglected associations between sponges and protists

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ABSTRACT

While bacterial and archaeal communities of sponges are intensively studied, given their importance to the animal's physiology as well as sources of several new bioactive molecules, the potential and roles of associated protists remain poorly known. Historically, culture-dependent approaches dominated the investigations of sponge-protist interactions. With the advances in omics techniques, these associations could be visualized at other equally important scales. Of the few existing studies, there is a strong tendency to focus on interactions with photosynthesizing taxa such as dinoflagellates and diatoms, with fewer works dissecting the interactions with other less common groups. In addition, there are bottlenecks and inherent biases in using primer pairs and bioinformatics approaches in the most commonly used metabarcoding studies. Thus, this review addresses the issues underlying this association, using the term "microeukaryome" to refer exclusively to protists associated with an animal host. We aim to highlight the diversity and community composition of protists associated with sponges and place them on the same level as other microorganisms already well studied in this context. Among other shortcomings, it could be observed that the biotechnological potential of the microeukaryome is still largely unexplored, possibly being a valuable source of new pharmacological compounds, enzymes and metabolic processes.

1. Introduction

The association between organisms is an ancestral and vital feature of the eukaryotic condition (Douglas, 2014). This intimate dependence is seen in the theories of origin of mitochondria, plastids and other organelles (López-García et al., 2017). Such an associative tendency is something that permeates most evolutionary lineages of Eukarya. In the group comprising animals (Metazoa) this is well observed in the marine environment (Fiore et al., 2010; German et al., 2011; Schippers et al., 2012; Vanwonterghem and Webster, 2020). In this context, being the first metazoans to diverge (Redmond and McLysaght, 2021), sponges have been widely studied in a variety of areas. In investigations of the interactions between microorganisms and animals, Porifera stand out as model animals (Hentschel et al., 2006; Taylor et al., 2007; Webster and Taylor, 2012, 2018). Initially, sponge-associated microorganisms were studied from a morphological viewpoint (*e.g.* Vacelet and Donadey, 1977; Wilkinson, 1978a, 1978b, 1978c, Wilkinson et al., 1984). To these classical works were added others focused on culture of selected strains which provided the basis for this area (*e.g.* Wilkinson et al., 1981; Padgitt and Moshier, 1987; Santavy and Colwell, 1990; Shieh and Lin, 1994; reviewed by Taylor et al., 2007).

With the advent and popularization of the omics approaches the communities of associated microorganisms, especially Bacteria and Archaea, have been further explored. This has provided advances such as the characterization of an enriched phylum of bacteria discovered in marine sponge samples (Poribacteria - Fieseler et al., 2004; Podell et al., 2019). Although omics approaches are applied with relative success to the symbioses of these ancient animals, there are still several gaps in scientific knowledge. Within the enormous biodiversity of the Porifera microbiome there is still a group that is little discussed. Fungi and other microeukaryotes form a significant portion of the sponge microbiome (Taylor et al., 2007; Suryanarayanan, 2012). In other invertebrates such

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as corals, these other symbionts can play an essential role in maintaining the animal's homeostasis as well as disrupting it (Ainsworth et al., 2017). In the recent years, sponge-associated fungi have gained some relevance mainly with regard to biotechnological applications (Suryanarayanan, 2012; Indraningrat et al., 2016; Zhang et al., 2020; Ben-Dor Cohen et al., 2021). However, our understanding of the role of microeukaryotes was and still is greatly hampered by the focus on bacterial and archaeal portions of the microbiome (Caron et al., 2009; Keeling and del Campo, 2017). Furthermore, when it comes to the association between Porifera and unicellular eukaryotes many of the records are focused on a few taxa such as diatoms (Cox and Larkum, 1983; Bavestrello et al., 2000; Regoli et al., 2004; Moitinho-Silva et al., 2017) and dinoflagellates (Garson et al., 1998; Schönberg and Loh, 2005; Schönberg and Suwa, 2007; Müller et al., 2007; Achlatis et al., 2019).

Overall, the current understanding of unicellular eukaryotic microorganisms has grown. Expeditions such as Tara Oceans shed light on this much under-explored groups (de Vargas et al., 2015; Delmont et al., 2022). There is also a focus on the questions related to biomass. It is estimated that marine unicellular eukaryotes can make up approximately 2 gigatons of carbon (Bar-On et al., 2018). Such immensity of microorganisms from a functional-ecological point of view becomes indispensable for the various biogeochemical cycles of the planet (Worden et al., 2015). Considering the importance of protists in aquatic environments in general and the "*future directions in sponge holobiont research*" defined by Pita and collaborators (2018), we bring in this text a narrative analysis of the relevant scientific literature about these microorganisms in the sponge holobiont. We seek to demonstrate that marine and freshwater sponges have associations with various protist lineages and that these have important functions (Fig. 1). For instance, processes such as photosynthesis are performed by diatoms, dinoflagellates and green algae, where there is an exchange of photosynthates for the protection and use of nitrogen wastes. Other phenomena such as syntrophy are reasonable to expect within a complex community. In addition to that, the production of secondary metabolites is also one of the main services provided by these unicellular eukaryotes. Compounds such as antimicrobials, fatty acids, and alkaloids produced by these microorganisms can contribute to the biochemical profile of the sponge (Indraningrat et al., 2016; Zhu et al., 2019; de Kluijver et al., 2021). Also, mechanisms such as top-down pressure exerted on the bacterial, archaeal, fungal and viral community modulate the structure and composition of these assemblages.

We understand that the definitions of eukaryome made by Lukeš et al. (2015) and del Campo et al. (2020) are more wide-ranging than we intend to address. Therefore, we define the term "microeukaryome" as the community of microorganisms that comprises only single-celled eukaryotes known as protists. Unicellular and multicellular fungi have not been included in this definition because we consider the term mycobiome/mycobiota to encompass this diversity (Peay et al., 2016), it is already used in the context of spongiology and recently reviewed (Bovio et al., 2020; Ben-Dor Cohen et al., 2021). From this definition we have also excluded any macroeukaryotes such as animals, plants or macroalgae. Our goal is to highlight the diversity and community composition of protists associated with sponges and place them on par with other already well-studied microorganisms in the holobiont context. Thus, here we present the taxa that have already been reported associated with sponges, a concise discussion of the possible physiological roles of these associated unicellular eukaryotes and their putative biotechnological potentials. In addition, we discuss the major obstacles in researching this important group and possible lines of investigation that may contribute to the solution of the problems presented.

2. A brief discussion on the study and phylogeny of eukaryotes

Before discussing the protist diversity associated with sponges, we should pay special attention to the eukaryotic diversity in general. Much is known about macroscopic lineages such as animals, fungi and plants. However, considering the myriad of habitats on the planet, we will see that in addition to bacteria, archaea and viruses, protists are essential in several of these spaces (Worden et al., 2015). In general, there are two main problems in the study of unicellular eukaryotes: cell size and

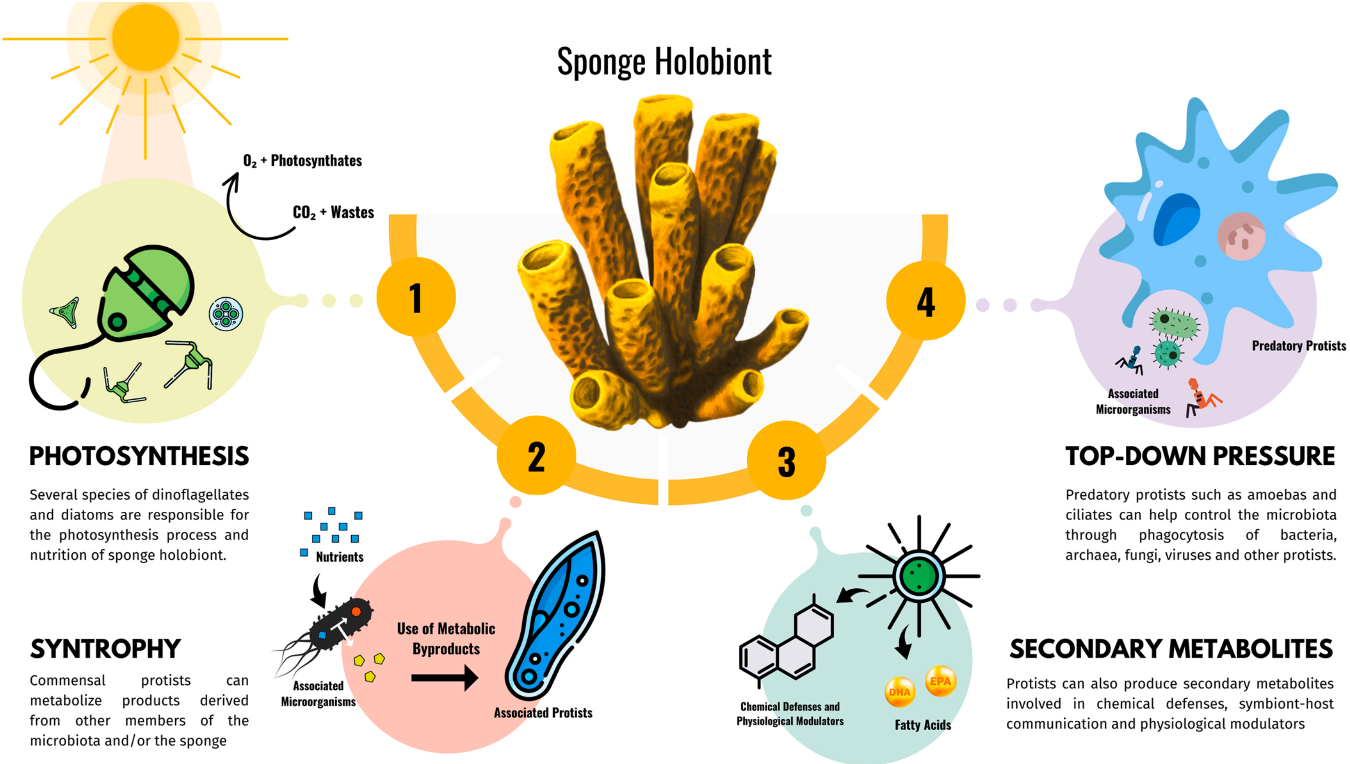

Fig. 1. Putative microbial services provided by the Porifera microeukaryome.

lineage diversity (Keeling and del Campo, 2017). The first concerns the inherent difficulty in visualizing, culturing and studying protists. Although methodological advances have mitigated these problems there are still obstacles to overcome. One such major challenge is the isolation and culture of uncultured protist strains, of which we have only a glimpse of knowledge about their genomes, obtained mainly through culture-independent approaches. The second major problem in protistology refers to the diversity of each strain itself. This characteristic is still difficult to be fully understood even with the molecular and bioinformatics tools currently available. This is mainly due to the absence of reference genomic sequences with which we can compare microeukaryotic sequences obtained to generate more robust assemblies (Dawson and Hagen, 2009; Keeling and del Campo, 2017). Furthermore, in the field of protistology there is a strong tendency to apply the same metabolic-functional inference-based strategies used in Bacteria and Archaea (Keeling and del Campo, 2017). Such an approach creates some methodological problems, since microeukaryotes in general have a relative metabolic homogeneity compared to the other two domains (Keeling and del Campo, 2017). In this context, morphology and behavior can be more informative features than inferences based only on DNA and RNA sequences (Keeling and del Campo, 2017; Keeling, 2019).

Regarding the relationships between lineages, it can be said that the phylogeny of the group is highly dynamic (Baldauf, 2003; Keeling et al., 2005; Roger and Simpson, 2009; Hampl et al., 2009; Adl et al., 2019; Burki et al., 2020). For simplicity, we adopt here a version made from the detailed review by Adl et al. (2019) and Burki et al. (2020), since it is beyond the scope of this review to detail the positioning of specific groups. In a comprehensive overview of the major evolutionary lineages within the tree of eukaryotes (Fig. 2), one can see the division into two major groups: Amorphea and Diaphoretickes. In the former there is the clades of Amoebozoa, Nucletmycea and Holozoa, which have as their best-known members the amoebas, true fungi and animals, respectively. Importantly, this hypothesis incorporates recent evidence demonstrating the paraphyly of Excavata that are now divided into clades such as Metamonada and Discoba (Adl et al., 2019; Burki et al., 2020). In Diaphoretickes, on the other hand, are the photosynthesizing organisms. It contains part of the former taxon Excavata, represented by algae of the taxon Cryptista. Still in Diaphoretickes there are Archeoplastida, comprising the red algae Rhodophyceae, the green algae Chloroplastida, and Embryophyta, if land plants are included. The SAR supergroup is monophyletic, composed of three other major clades: Stramenopiles, Alveolata and Rhizaria. To facilitate the understanding of their associations with Porifera, Alveolata is represented here divided into smaller groups. From the limited literature on the associations of Porifera and microeukaryotes, we observed two major trends on the study of the biodiversity of these sponge-associated microorganisms. The first concerns research that was able to study these associations through direct observations and classical isolation and culture methods. Another more recent approach makes use of molecular methods and bioinformatics tools to investigate the diversity of these protists in the sponge holobiont.

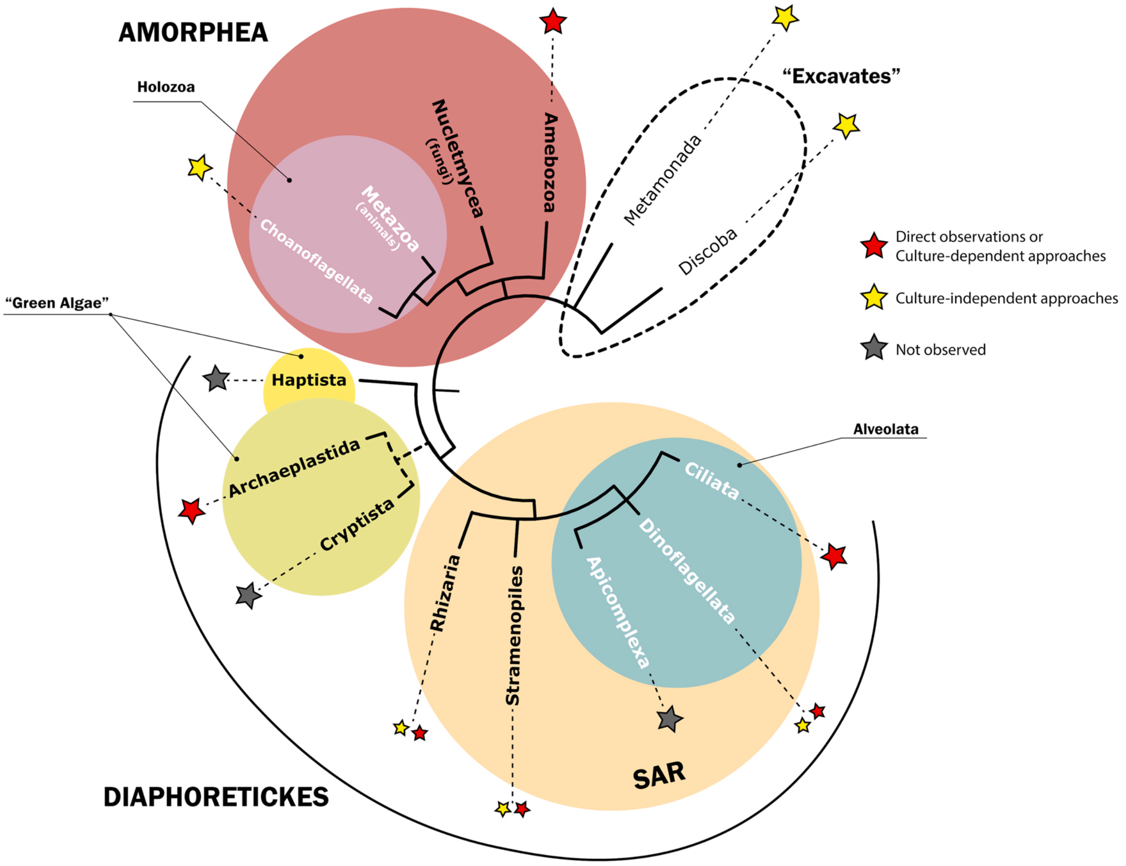

Fig. 2. Simplified scheme of the main evolutionary lineages of Eukarya based on Adl et al. (2019) and Burki et al. (2020). Amorphea is the group that includes true animals and fungi. Within this clade we have here as representatives of Holozoa the protists of Choanoflagellata as a sister group to the animals or Metazoa. In turn, Diaphoretickes is an extremely diverse supergroup. Within it is the SAR supergroup, consisting of Stramenopiles, Alveolata and Rhizaria. There is also Archaeplastida, which contains part of the diversity of multicellular and unicellular protists, as well as land plants. This group and the Cryptista protists have widely debated monophyly. The 'green algae' are a paraphyletic group that is generally composed of representatives of these two groups plus Haptista. Excavata, here represented by the Metamonada and Discoba clades is no longer considered monophyletic and these groups have uncertain positioning. There are more protist lineages that are not represented in this figure.

3. The microeukaryome diversity from direct observations to culture-dependent approaches

The culture-dependent approaches allow the elucidation of features not visualized in gene sequences such as structure, metabolism and behavior (Keeling and del Campo, 2017; Konstantinou et al. 2020; Škodová-Sveráková et al., 2021). However, it is limited to a few microorganisms, implying a low resolution of the biodiversity of host-associated microeukaryotes in a manner similar to "the great plate count anomaly" first described by Staley and Konopka (1985). Despite the enormous biodiversity of microorganisms on the planet, only a small portion can be grown in the laboratory for diverse reasons (Epstein, 2013; Cross et al., 2019) one of them being the symbiont-host interdependence itself (Kikuchi, 2009; Hendry et al., 2014; Fisher et al., 2017).

There are few studies that actively seek to understand in detail the nature of sponge-protist associations. Although there is research that focus on these interactions, the isolation and culture of these microeukaryotes in the vast majority of cases occurs indirectly and unintentional. For example, in primary host cell cultures contaminant protists are one of the main problems. They benefit from the dysbiosis caused by the stress of host cell reorganization and proliferate, often outgrowing the sponge cells. The diversity of unicellular eukaryotes makes it difficult to control the growth of their populations in primary cell cultures of marine invertebrates, a problem that diminishes when the contaminant is a bacterium or fungus, given the large number of commercially available antimicrobials. Many sponges primary cell cultures become non-viable after a short time of establishment when contaminated by some species of protists. The almost constant presence of these microorganisms is one of the major obstacles to be overcome for the establishment of axenic cell lines of Porifera and several other marine invertebrates (Rinkevich, 1999; Grasela et al., 2012; Schippers et al., 2012; Nowotny et al., 2021; Qarri et al., 2021). Here, we attempt to synthesize from the literature the main records of isolations and culture of non-fungal microeukaryotes associated with marine and freshwater sponges. A comprehensive list of several taxa of unicellular eukaryotes associated with sponges can be found in Table 1.

3.1. *Amoebozoa*

Amoebas are present in diverse habitats ranging from soils, mangroves, lakes and oceans (Rodríguez-Zaragoza, 1994; Rogerson and Gwaltney, 2000; Schulz et al., 2015). Along with other protists they are important predators of other microorganisms such as bacteria, archaea and viruses. The taxon Amoebozoa is characterized by amoeboid movement, which consists of altering cell conformation through pseudopod extensions and contractions (Adl et al., 2019). Common in primary Porifera cell cultures, they are reported as persistent contaminants, where they assume opportunistic behavior (Rinkevich, 1999). There are few reports of associations between amoebae and sponges and, as for the vast majority of unicellular eukaryotes, the diversity of associations is poorly studied. The amoeba *Neoparamoeba aestuarina* (Discosea) was isolated from dissociated tissues of *Clathrina aurea, Polymastia janeirensis* (Klautau et al., 1993, misidentified as sponge cells) and *Chelonaplysilla erecta* (Custodio et al., 1995). In another work with adult tissues and embryos of *Negombata* spp. several species of amoebae proliferated, but were not identified (Rinkevich et al., 1998; Rinkevich, 1999). These microorganisms have already been isolated from other algae, invertebrates and fishes, where they appear to play negative effects, such as Amoebic Gill Disease in marine farmed fishes (Dyková et al., 2007, 2008). It is still uncertain whether the presence of amoebas in sponges characterizes some kind of parasitism or their proliferation is a result of opportunistic behavior. The amoebae, in general, are free-living and from this we can infer that the persistence of these microorganisms in cultures is due to the abundance of bacteria that the sponges harbor. They may play a role of predators of the microbial community (Fig. 1), thus contributing to the control of the population of sponge-associated microorganisms. Such a function is reported in other symbiotic systems, such as corals (Ainsworth et al., 2017) and plants (Gao et al., 2019).

3.2. *"Green algae"*

The term "green algae" is the common name for several unicellular and multicellular photosynthesizing eukaryotic organisms. Despite the lack of phylogenetic consensus this group comprises some organisms of the taxon Archeoplastida, Cryptista and Haptista (Simpson et al., 2017; Adl et al., 2019). Multicellular organisms are represented by macroalgae and land plants, but a significant part of the diversity is composed by unicellular microorganisms. With an emphasis on the latter, it is noted that these microeukaryotes are relatively common as epibionts and endobionts of Porifera. Some of these associations have been used as a model for understanding the general process of establishing intracellular symbioses, such as in the freshwater sponge *Ephydatia muelleri* and its *Chlorella*-like symbionts (Hall et al., 2021; Geraghty et al., 2021). From the interactions between sponges and algae, several benefits can be derived for both participants, the main ones being obtaining oxygen, nutrients and recycling of metabolic wastes (Trautman and Hinde, 2002).

One of the most studied associations is the symbiosis between the freshwater sponge *Spongilla lacustris* and the algae of the genus *Chlorellae*. The greenish color of this sponge is primarily due to their association with these algae. The first morphological works that sought to characterize this symbiosis date back to the 1910s and 1920s (Williamson, 1979; Saller, 1989). Also called zoochlorellae, these algae are often found in the tissues of adult sponges, within the vacuoles of archaeocytes and other cell types as well as in the gemmules (Williamson, 1979; Saller, 1989). The presence of the symbionts protists in reproductive structures is evidence of the vertical transmission between generations. Still in the freshwater environment, the symbiosis between *Lubomirskia baicalensis* and endosymbiont green algae of the genus *Mychonastes* was described in Lake Baikal (Chernogor et al., 2013). Through the method of cultivation of primmorphs of this animal, it was possible to isolate and subsequently cultivate these symbionts. Contrary to the trend observed for other freshwater sponge species, *Lubomirskia baicalensis* is perennial even in the face of harsh Siberian winter conditions, maintaining its association with its symbionts (Müller et al., 2009; Chernogor et al., 2013). It is suggested that *Mychonastes* plays an important role in the nutritional homeostasis of its host. The sponge would provide a safe and resourceful environment (shelter from predation and metabolic wastes) for the proliferation of the protist, which reciprocates through photosynthates and derivatives such as oxygen (Chernogor et al., 2013). It is also plausible to consider that this association between these green algae and the sponge has other benefits for the host, mainly in the form of protection against UV radiation through the production of mycosporine-like amino acids, as has been seen in several taxa of photosymbionts (reviewed by Carreto and Carignan, 2011). It is worth noting that the sponge-algae association is maintained even during the organizational change of the host tissues into primmorphs. This intimate symbiosis might be one of the main responsible for the long-term maintenance of primmorphs under laboratory conditions - over 10 months - without the addition of nutrients in the culture medium (Chernogor et al., 2011, 2013). Another interesting symbiosis occurs between the marine sponge *Tethya seychellensis* and the coenocytic green algae *Ostreobium* sp. (Gaino and Sarà, 1994). Algae of this group are commonly found in association with other sessile invertebrates such as ascidians (Lambert et al., 1996), barnacles (Feussner et al., 2004), corals and gorgonians (del Campo et al., 2017). Organized in a globular shape, sponges of the genus *Tethya* have large siliceous spicules bundles arranged radially along their body. Such organization favors the growth of the associated algae from the cortex to the most nuclear region of the animal's body (Gaino and Sarà, 1994). It was speculated that, because they are mostly arranged around the spicular

Table 1
Comprehensive list of protists associated with marine and freshwater sponges.

Taxon	Protist ID or Most Abundant Protist Taxa	Sponge-host	Location	Study type	Reference
Amoebozoa	Non-identified amoeba	*Negombata* sp.	Red Sea (Israel)	Culture dependent (contaminant)	Rinkevich et al. (1998)
	Neoparamoeba aestuarina	*Clathrina aurea* *Polymastia janeirensis* *Chelonaplysilla erecta*	Rio de Janeiro Coast (Brazil)	Observation only Culture dependent	Klautau et al. (1993) Custodio et al. (1995)
"*Green Algae*"	*Chlorella*-like	*Ephydatia muelleri*	Richmond, Virginia (USA)	Culture dependent	Hall et al. (2021) Geraghty et al. (2021)
	Chlorellae	*Spongilla lacustris*	Massachusetts (USA) Sieg River (Germany)	Observation only Culture dependent	Williamson (1979) Saller (1989)
	Mychonastes sp.	*Lubomirskia baicalensis*	Lago Baikal (Russia)	Culture dependent	Chernogor et al. (2013)
	Ostreobium	*Tethya seychellensis*	Ari Atoll (Maldive Islands)	Observation only	Gaino and Sarà (1994)
Stramenopiles	*Nitzschia* spp. (Diatom)	*Batzella melanus* *Spirastrella* aff. *decumbens*	One Tree Island (Australia)	Observation only	Cox and Larkum (1983)
	Fragilariopsis spp. (Diatom) *Achnanthes* spp. (Diatom) *Pseudogomphonema* spp. (Diatom)	17 marine sponges species	Terra Nova Bay (Ross Sea)	Observation only	Bavestrello et al. (2000)
	Porannulus contentus (Diatom) *Thalassiosira* cf. *gracilis,* (Diatom) *Thalassiosira. Perpusilla* (Diatom) *Plagiotropis* sp. (Diatom)	*Mycale acerata*	Terra Nova Bay (Ross Sea)	Observation only	Cerrano et al. (2004)
	Diatomea	*Aplysina caissara* *Aplysina fulva* *Tedania ignis*	São Paulo Coast (Brazil)	Culture independent	Hardoim et al. (2021)
	Unidentified Labyrinthulomycetes	*Xestospongia muta*	Caribbean Sea (Curaçao)	Culture dependent (contaminant)	Richelle-Maurer et al. (2003)
		Negombata sp.	Red Sea (Israel)	Culture dependent (contaminant)	Rinkevich et al. (1998)
	Ulkenia visurgensis (Labyrinthulomycetes) *Oblongichytrium multirudimentale* (Labyrinthulomycetes)	*Geodia cydonium* *Aplysina sp.* *Ircinia fasciculata*	Ischia (Italy)	Culture dependent	Höhnk and Ulken (1979)
Alveolata	*Lagenophrys* (Ciliophora)	*Swartschewskia khanaevi*	Lago Baikal (Russia)	Culture dependent	Bukshuk and Maikova (2020)
	Non-identified ciliates (Ciliophora)	*Petrosia ficiformis*	Island of Gallinara (Italy)	Observation only	Cerrano et al. (2001)
	Symbiodinium spp.	*Cliona* spp.	Blanes (Spain) Florida Keys (USA) Heron Island (Australia) Okinawa (Japan)	Observation only Culture independent	Mariani et al. (2001) Schönberg and Loh (2005) Hill et al. (2011)
	Symbiodinium microadriaticum	*Haliclona* sp.	Heron Island (Australia)	Culture dependent	Garson et al. (1998)
	Gymnodinium sp.	*Lubomirskia baicalensis*	Lago Baikal (Russia)	Culture dependent	Müller et al. (2007) Müller et al. (2009)
	Dinophyceae (Dinoflagellata)	Several sponge species	Antarctic Sea São Paulo coast (Brazil)	Culture independent	Rodríguez-Marconi et al. (2015) Hardoim et al. (2021)
	Syndiniales (Dinoflagellata)	Several sponge species	Antarctic Sea Penghu Archipelago (Taiwan)	Culture independent	Rodríguez-Marconi et al. (2015) Cleary (2019)
	Suessiales (Dinoflagellata)	*Baikalospongia intermedia* *Baikalospongia recta* *Lubomirskia incrustans*	Lake Baikal (Russia)	Culture independent	Annenkova et al. (2011)
Rhizaria	Radiolarians	Several sponge species	Chinese Sea (China) Penghu Archipelago (Taiwan)	Culture independent	He et al. (2014) Cleary (2019)
	Lotharella oceanica (Cercozoa)	*Hexadella racovitzai*	Chalkidiki Peninsula, Aegean Sea (Greece)	Culture dependent	Konstantinou et al. (2020)
	Cibicides refulgens (Foraminifera) *Hyrrokkin sarcophaga* (Foraminifera)	*Geodia phlegraei* *Stelleta normani*	Faroe Islands Archipelago	Observation only	Cedhagen (1994) Klitgaard (1995)
	Placopsilina spongiphila (Foraminifera) *Ramulina siphonifera* (Foraminifera)	Fossil Sponge Reefs	British Columbia (Canada)	Observation only	Guilbault et al. (2006)
Choanoflagellata	Choanoflagellida	*Dysidea avara* *Dysidea etheria* *Aplysina aerophoba* *Aplysina cauliformis*	Florida Keys (EUA) Mediterranean Sea (Spain)	Culture independent	De Mares et al. (2017)
Metamonada	Metamonads	*Acanthostylotella cornuta*	Penghu Archipelago (Taiwan)	Culture independent	Cleary (2019)

bundles of the host, the algae benefit from a light conduction system similar to an optical fiber (Gaino and Sarà, 1994). The transport of photoactive radiation by spicules was corroborated more than a decade later using *Tethya aurantium* (Brümmer et al., 2008).

3.3. SAR: Stramenopiles

The Stramenopiles, like the other SAR groups, constitute a very diverse lineage. Some of its more derived representatives are photosynthesizing while the more basal forms are heterotrophic. This group has as its main characteristic the presence of tubular flagella with tripartite hairs in at least one of the stages of its life cycle (Grattepanche et al., 2018). In Stramenopiles are contained well-known groups such as the "brown algae" (Phaeophyceae) and the diatoms (Diatomea). The diatoms are one of the groups that have associations with various invertebrates, including intimate symbioses with marine sponges. There are records of the association between some unidentified tropical sponges, possibly *Prianos* cf. *melanos* (now accepted as *Batzella melanus*) and *Spirastrella* aff. *decumbens,* and the diatoms of the genus *Nitzschia* (Cox and Larkum, 1983; Cox et al., 1985). Although there is no physiological approach to this association, the sponge-diatom relationship appears to be stable, as it has been observed in more than one locality (Cox and Larkum, 1983). A large number of these protists have been reported living extracellularly in the mesohyl of the sponge, with their frustules intact. On the other hand, Antarctic sponges host a large diversity of diatoms in their mesohyl, just below the pinacoderm (Gaino et al., 1994; Bavestrello et al., 2000). In 17 Antarctic sponge species, diatoms of the genus *Fragilariopsis*, *Achnanthes* and *Pseudogomphonema* were the most commonly found, followed by some species of centric diatoms (Bavestrello et al., 2000). Although more evidence is still needed, it is hypothesized that these microeukaryotes strengthen the cortex of the animal and the accumulation of frustules within the choanosome during the cold season indicates the possibility that they serve as nutritional reservoirs in the harsh Antarctic conditions (Gaino et al., 1994; Cerrano et al., 2004). In exchange, these associated diatoms appear to benefit from metabolic wastes of the host, adopting a myxotrophic strategy that allows survival in low or no light situations (Bavestrello et al., 2000).

In addition to photosynthesizing organisms, there are also associations between Porifera and heterotrophic stramenopiles. Of this subgroup, the interactions of sponges with the Labyrinthulomycetes, a small group of saprophytic, fungi-like microeukaryotes, stand out. Overall, their relationships with marine invertebrates and other organisms tend to be negative, with significant impacts to host fitness (Raghukumar, 2002; Schärer et al., 2007; Gast et al., 2008). However, it is likely that these microorganisms are opportunistic, benefiting from an eventual lack of regulation of the microbiota by the host. Yet, there is evidence that they are important at times of stress, such as in bleaching events on stony corals of the genus *Fungia* (Kramarsky-Winter et al., 2006). From the few studies that exist on the interaction of these protists with sponges, it is noted that they can be isolated from tissues derived both from healthy and decaying specimens (Höhnk and Ulken, 1979; Raghukumar, 2002). They are also recurrently isolated from primary cell cultures, probably benefiting from the dysbiosis caused by the tissue dissociation. With rapid proliferation, these unicellular eukaryotes can be mistaken for well-established Porifera cultures (Rinkevich, 1999; Richelle-Maurer et al., 2003). The first research conducted that demonstrates the association of these protists with Porifera dates back to the late 1970s (Höhnk and Ulken, 1979). Through culture-dependent approaches and using the tissues of the marine sponges *Geodia cydonium*, *Aplysina* sp. and *Ircinia fasciculata* the authors isolated two species of Labyrinthulomycetes now classified as *Oblongichytrium multirudimentale* and *Ulkenia visurgensis*. Although they did not delve into the physiology of this association, it was demonstrated that they can be consistently isolated and cultivated (Höhnk and Ulken, 1979). It is possible that these microorganisms adopt endo- and exosymbiont strategies, similarly to diatoms. Their ectoplasmic projections, a synapomorphy of the group, may facilitate the uptake of metabolic wastes from the host, as well as particulate and dissolved organic matter. They are recognized producers of polyunsaturated fatty acids (PUFAs), carotenoids, polysaccharide exudates, squalene, and enzymes with varied metabolic activities (Marchan et al., 2018). Although further investigations are still needed on the role of this rich diversity of metabolites and enzymes in the association between Labyrinthulomycetes and Porifera it is possible to infer that part of the carotenoid profile of these animals (Liaaen-Jensen et al., 1982) may come from these protists. Similarly, there can be translocation of PUFAs between these microeukaryotes and their hosts, especially ω-3 and ω-6 which are important metabolites in the sponge's stress response to the new environmental challenges brought by climate change (Bennett et al., 2018). These metabolites can also be used in the structure of cell membranes, precursors of immunomodulators and the reproduction of these animals, being important in the formation of the yolk of oocytes and embryos (Bennett et al., 2018; Koutsouveli et al., 2022).

3.4. SAR: Alveolata

Alveolata is a monophyletic group of protists with alveolar sacs, consisting of three major taxa (Apicomplexa, Dinoflagellata and Ciliophora) (Grattepanche et al., 2018). It comprises microorganisms with diverse life habits. One of the best known, mainly for its negative impacts on host fitness, is the Apicomplexa. This lineage has as its most distinguishable feature a cellular structure known as the apical complex (Katris et al., 2014). However, this direction to the study of parasites may be the result of a strong bias as there is an unexpected biodiversity yet to be explored (Saffo et al., 2010; Oborník et al., 2012; del Campo et al., 2020; Keeling et al., 2021). Previous studies of isolation and identification of Apicomplexa from Porifera tissues or cell cultures are unknown to us. In contrast, there are records of Porifera-Ciliophora and Porifera-Dinoflagellata interactions as a focus. The taxon Ciliophora is widely studied in the free-living context (Simpson et al., 2017) and as model organisms in various areas of science (Abraham et al., 2019). They have nuclear dimorphism as their main characteristic, possessing two functional cell nuclei: a macronucleus and a micronucleus (Simpson et al., 2017; Abraham et al., 2019).

In the recently described Lake Baikal demosponge, *Swartschewskia khanaevi*, the presence of sessile epibiont ciliates of the genus *Lagenophrys* was observed (Bukshuk and Maikova, 2020). It is possible that the proliferation of these protists, along with diatoms that also make up the microeukaryome of these animals, is favored by the particular morphology of the sponge's aquiferous system (Bukshuk and Maikova, 2020). In environmental stress events, which usually result in tissue necrosis and possible mass mortality for sponges, ciliates can play important roles. In a study of *Petrosia ficiformis* populations, it was observed that these ciliates consume the degenerated tissues of the sponges, allowing the animals to recover from a necrosis event (Cerrano et al., 2001).

Dinoflagellata are recognized symbionts of invertebrates and have as one of their distinguishing characteristics the presence of cells with two cilia in the motile phase and permanently condensed chromosomes that are devoid of nucleosomes (Grattepanche et al., 2018). In terms of Porifera-Dinoflagellata interaction one of the most studied associations occurs between excavating sponges of the genus *Cliona* and various *Symbiodinium* clades (Schönberg and Loh, 2005; Hill et al., 2011; Achlatis et al., 2019). This association can be destabilized in a manner similar to what occurs in coral-*Symbiodinium* symbioses, as was reported for the first record of mass bleaching in clionaid sponges in 2015 (Hill et al., 2016). There is even evidence of vertical transfer of these symbionts between generations of *Cliona* sp. (Mariani et al., 2000, 2001; Schönberg and Loh, 2005). Interestingly, this association has recently been included, although briefly addressed, in a broader discussion that proposes a new understanding of the acquisition of symbionts by sponges (de Oliveira et al., 2020a; Carrier et al., 2022). Besides the metabolic exchange between these symbiont partners, there are other benefits that *Symbiodinium* clades can provide to the host such as protection against UV radiation and response mechanisms to reactive oxygen species (González-Pech et al., 2017). Although this is the best-known association, there are other investigations that are worth highlighting. Through an ultrastructural and morphological approach, it was suggested that one Haplosclerida, collected on the Great Barrier Reef and identified as *Haliclona* sp., contains a dinoflagellate very similar to *Symbiodinium microadriaticum* in its tissues (Garson et al., 1998). In Lake Baikal, the sponge *Lubomirskia baicalensis* and a dinoflagellate species of the genus *Gymnodinium* have a well-characterized symbiosis, mainly from a physiological point of view. Besides providing the hosts with primary metabolites, such as glycerol, there is evidence of their participation in the acclimatization of sponges (Müller et al., 2007, 2009). During the winter, Lake Baikal is covered by a layer of ice and it is at this time that a higher metabolic activity related to the reproduction of these sponges is observed (Müller et al., 2007). It has been identified that these symbiont dinoflagellates produce a secondary metabolite called okadaic acid. This molecule interacts with the host HSP70 system, increasing the expression of heat-shock proteins and consequently contributing to the survival of these animals in the harsh winter conditions of Lake Baikal (Müller et al., 2007). It is interesting to note that the interaction between sponges and protists that produces okadaic acid are studied since the 1980s. Okadaic acid was first isolated from associated microorganisms of two sponges: *Halichondria okadai* and *Halichondria melanadocia* (Tachibana et al., 1981). This molecule, classified as an ionophore, exhibits a number of characteristics that attracted strong academic and commercial interest over time. The compound was the first described substance capable of reversibly inhibiting type 1 and type 2 phosphatases (Bialojan and Takai, 1988). Therefore, it became an important tool in the investigation of various cellular processes such as smooth muscle contraction, synaptic plasticity, tumor formation and neurotoxicity (Fernandez et al., 2012; Kamat et al., 2014).

3.5. SAR: Rhizaria

Representatives of Rhizaria form a morphologically diverse group. These microorganisms have as their main characteristic thin pseudopodia, which display varying patterns, from simple extracellular projections to the ones with anastomosed and branched aspects (Simpson et al., 2017; Grattepanche et al., 2018). Because of its great diversity, there is no universal set of morphological characteristics that support the group, but phylogenetic relationships among the taxa can be obtained from molecular studies (Grattepanche et al., 2018). Within Rhizaria are three other prominent groups: Radiolaria, Cercozoa and Foraminifera. While there is no record of the use of culture-dependent approaches in examining the association between radiolarians and sponges, the interactions of cercozoans and foraminifera with Porifera are relatively better known.

Recently, Konstantinou et al. (2020) isolated and cultured a photosynthetic cercozoa, identified as *Lotharella oceanica*, associated with the sponge *Hexadella racovitzai*. This is the first described association between a sponge and a chlorachniophyte. It is important to highlight that the morphological observations made by the authors did not entirely match the description of the species and it was hypothesized that the microenvironment provided by the sponge caused changes in the morphology of this protist (Konstantinou et al., 2020). Being a photosynthesizing microorganism, it is therefore expected that an exchange of nutrients and photosynthates occurs between *Lotharella oceanica* and its host sponge *Hexadella racovitzai*, similar to other associations already described between sponges and other photosynthesizing protists such as dinoflagellates, diatoms and “green algae”.

Foraminifera are important in biogeochemical cycles, as they have shells or tests composed of calcium carbonate, aragonite, agglutinated particles in an organic matrix or opaline silica (as is the case of the genus *Miliamellus*) (Tappan and Loeblich, 1988; Pawlowski et al., 2013). These protists adopt myxotrophic strategies and are often epibionts of sponges, but there are also records of being inside the body of these animals (Granville and Nordmann, 1971; Linke and Lutze, 1993; Mazzoli-Dias et al., 2007). In a survey of the Faroe Islands archipelago, these protists were found in the pinacoderm of all studied sponges of the family Geodiidae (Klitgaard, 1995). The most common species were *Cibicides refulgens* and *Hyrrokkin sarcophaga*, the latter described as a parasite of corals and bivalves (Schleinkofer et al., 2021). Histological approaches have shown that in the sponges *Isops phlegraei* (now accepted as *Geodia phlegraei*) and *Stelleta normani,* the foraminifera *H. sarcophaga* can dissolve part of the cortex of the animal (Cedhagen, 1994). In turn the host's spicules are used by the protist, which deposits them on its external part. The outcome of this interaction is a negative impact on the sponge, left with scars where the spicules were removed (Cedhagen, 1994). Despite these observations, further investigation of the physiology of this interaction is still needed. It is possible that *H. sarcophaga*, like other protists, utilize the sponge body for reproduction and ease of obtaining nutrients, as they have been found at different stages of development within the hosts (Lintner et al., 2021). The association between foraminifera and sponges appears to be an old one. Several species of foraminifera already known to science and two new species - *Placopsilina spongiphila* and *Ramulina siphonifera* - have been identified in fossil sponge reefs (Guilbault et al., 2006). Another interesting aspect of Foraminifera-Porifera interaction is starred by the giant Mediterranean Sea foraminifera *Spiculosiphon oceana*. Through a meticulous organization of siliceous sponge spicules, this organism builds its test or outer shell and assumes a feeding strategy and morphology very similar to carnivorous sponges (Maldonado et al., 2013).

4. The microeukaryome diversity from culture-independent approaches

The use of omics approaches has completely transformed our understanding of the microbial ecology of multiple sponge species, allowing a more comprehensive view. The integration of different approaches has shown that sponges and their associated microorganisms emerge as one of the most complex holobionts known (Pita et al., 2018). Metabarcoding allows us to know in detail the diversity, abundance and composition of entire communities of associated microorganisms in a generalized or group-specific manner. Similarly, metagenomics can provide information on the functional capacities of associate organisms, and also indicate important metabolic relations. Nevertheless, it alone does not allow the inference of morphological and behavioral characteristics which are important for the study of unicellular eukaryotes (Keeling and del Campo, 2017; Keeling, 2019).

Considering the Porifera microeukaryome, it is unknown to us the use of whole-metagenome shotgun sequencing and the metabarcoding approach started to be successfully applied only in the last decade, usually with sets of nonspecific primers for the 18S rRNA gene such as those listed in Table 2. One of the first studies applied pyrosequencing (now an almost unused technology) to study the communities of fungi and unicellular eukaryotes associated with 11 sponges sampled at the South China sea (He et al., 2014). In these species the diversity of protists was higher than that of fungi, with 721 unique Operation Taxonomic Units (OTUs) being obtained. Alveolata and Radiolaria were found in all investigated species and the protist sequences observed were significantly different from those in seawater, implying a host control on the composition of this community (He et al., 2014). In Antarctic sponges, diatoms (Diatomea) and dinoflagellates (Dinophyceae) are predominant in the microeukaryome (Rodríguez-Marconi et al., 2015). Although dominated by photosynthesizing microorganisms, heterotrophic dinoflagellates (Syndiniales) also constitute a significant portion of the protist community (Rodríguez-Marconi et al., 2015). However, in these two studies the authors did not use biological replicates and

Table 2

Primers used to study the diversity of unicellular eukaryotes and the 18S rRNA coverage of sponges. Data obtained from the TestPrimer tool (Klindworth et al., 2013) against the SILVA 138 RefNR database.

Primer pairs	Coverage of 18S rRNA of Porifera (%)	Coverage of 18S rRNA of Eumetazoa (%)	Region of 18S rRNA	Forward Primer (5'–3')	Reverse Primer (5'–3')	Sequencing technology	Reference
Euk1Af / Euk516r	97.70%	68.50%	V1-V3	CTGGTTGATCCTGCCAG	ACCAGACTTGCCCTCC	Next Generation Sequencing (Illumina)	De Mares et al. (2017)
TAReuk454FWD1 / TAReukREV3	91.10%	69.40%	V4	CCAGCASCYGCGGTAATTCC	ACTTTCGTTCTTGATYRA	Next Generation Sequencing (Illumina)	Cleary (2019)
EUK581-F /EUK1134-R	72.80%	1.80%	V4	GTGCCAGCAGCCGCG	TTTAAGTTTCAGCCTTGCG	Next Generation Sequencing (Illumina)	Bower et al. (2004) tested in del Campo et al. (2019)
18SV1V2F/ 18SV1V2R[#a]	–	–	V1-V2	ACCTGGTTGATCCTGCCA	GTARKCCWMTAYMYTACC	Next Generation Sequencing (Illumina)	Clerissi et al. (2018)
1391 f[#b] / EukBr	–	–	V9	GTACACACCGCCCGTC	TGATCCTTCTGCAGGTTCACCTAC	Next Generation Sequencing (Illumina)	Rodríguez-Marconi Hardoim et al., (2015, 2021)
574 *f/UNonMet DB	77.50%	1.90%	V4	CGGTAAYTCCAGCTCYV	CTTTAARTTTCASYCTTGCG	Next Generation Sequencing (Illumina)	Bass and del Campo (2020)

#a - The reverse primer 18SV1V2R has a number of 'wobbles' that cannot be interpreted and resolved using the TestPrime tool.

#**b -** The primer 1391 f is a universal primer, used for the three domains Archaea, Bacteria and Eukarya. Such a range, even when used in conjunction with a Eukarya-specific primer, does not allow a reliable analysis of the results on TestPrimer tool.

apparently did not normalize the data prior to alpha diversity metrics. Also, only Rodriguez-Marconi and collaborators included seawater samples in their experimental design. The approaches used in these studies directly impacts the understanding of the microeukaryotic community associated with these sponges, making comparisons difficult (Hardoim et al., 2021).

On the other side, these investigations have gathered further evidence that, just as occurs in the bacterial and archaeal communities, the protists associated with sponges can also possess host specificity. Nevertheless, this is not a consensus in the area. In a comparison between the microbiome of four sponge species from two different locations (*Dysidea avara* and *Aplysina aerophoba* from the Mediterranean Sea and *Dysidea etheria* and *Aplysina cauliformis* from the Caribbean Sea) no host specificity of the microeukaryome was observed (De Mares et al., 2017). Nonetheless, in this work there was a large variability in diversity among the samples of each species, possibly stemming from low sample numbers as pointed out by the authors. In the microeukaryome of these four sponges, the vast majority of reads could not be classified taxonomically. Of those that were classified, Arthropoda and Annelida followed by Choanoflagellida (protists) have the highest relative abundances (De Mares et al., 2017). A similar trend was seen when analyzing the microeukaryome of six sponge species native to the Penghu Archipelago in Taiwan. Much of the microeukaryotic community of these animals appears to be derived from the surroundings, primarily seawater (Cleary, 2019). Again, many organisms could not be classified into more specific taxa. This is a bias stemming from the little knowledge one has about protists in general; something that is present in many databases used and represents a strong indication of an unexplored diversity. Dinoflagellates (Dinophyceae and Syndiniales) were the most abundant group, but green algae and radiolarians were also present in remarkable proportions (Cleary, 2019). Interestingly, in the sponge *Acanthostylotella cornuta,* it was observed a high relative abundance of Metamonada, basal heterotrophic eukaryotes that are usually grazers of bacterial communities, but with parasitic representatives (Adl et al., 2019). In a more recent study (Hardoim et al., 2021), again the question of microeukaryome specificity was raised. Although the previous studies are not directly comparable given methodological divergences, the investigation of the microeukaryotic community of three sympatric species from the Southwestern Atlantic Coast indicates that the hosts directly act on the selection of microorganisms. The marine sponges *Aplysina caissara*, *Aplysina fulva*, and *Tedania ignis* have a high diversity of microeukaryotes that could not be taxonomically classified, indicative of microbial novelty. Of the identifiable taxa, the photosynthesizing groups were the most abundant, dominated by macroscopic green (Phragmoplastophyta) and red (Florideophycidae) algae, followed by diatoms (Diatomea) (Hardoim et al., 2021).

The second type of investigation, which considers only one or a few protists taxa, has also been used for Porifera. For example, a great diversity of dinoflagellates (Alveolata: Dinoflagellata) were found in the freshwater sponges *Baikalospongia intermedia*, *Baikalospongia recta* and *Lubomirskia incrustans* (Annenkova et al., 2011). Phylogenetic analyses in this work showed that the 18S rRNA of two groups of dinoflagellates were repeatedly found in the total DNA extracted from these sponges. The protists belong to the order Suessiales, a group comprising endosymbiont dinoflagellates known to be associated with a wide range of marine invertebrates, and may be a potential symbiont of the sponges in this ecosystem (Annenkova et al., 2011). However, many of these works are early stages that have not yet delved into the physiology of the association. The use of omics approaches (metagenomics and metatranscriptomics) that goes further and attempts to integratively understand several symbiotic aspects, function and metabolism of the sponge holobiont were applied by Moitinho-Silva and colleagues (2017). Within the marine sponge *Cymbastela concentrica* there is a complex network of metabolic integration where diatoms, bacteria, archaea, and the host cells themselves are the key players. The results of this work show that there is a transfer of carbon and nitrogen compounds between these participants, which supports the nitrogen cycle within the holobiont. In a simplified manner, the physiological interaction model developed by the authors indicates that the symbiont diatoms of *C. concentrica* (identified only as belonging to the Bacillariophyta) have much of the transcripts related to photosynthate production and consumption via glycolysis, which produces metabolic waste products (Moitinho-Silva et al., 2017). The dissolved organic carbon produced by this protist along with DOM from the sponge's filtering activity and nitrogenous compounds are mainly used by a sponge-associated bacterium of the family Phyllobacteriaceae. However, this bacterium together with other microorganisms of the genus *Nitrospira* and the order Nitrosopumilales, assimilate and produce nitrogenous compounds derived from the cellular metabolism of the holobiont, recycling these metabolites in a complex metabolic interplay, which are reused as is the case of the nitrite (NO_3^-) assimilation process used by the diatom associated with *C. concentrica* (Moitinho-Silva et al., 2017).

5. The problems of sequencing and analysis of the protists associated with Porifera

When dealing with the ocean of biological information there is a certain tendency to little question the assumptions and functioning of the main bioinformatics methods and tools used and the biases introduced by them (Keeling and del Campo, 2017). In this section we briefly discuss some of the main problems underlying the sequencing and analysis process of the sponge microeukaryome.

5.1. The choice of 18S rRNA primers for metabarcoding studies

The phylogenetic marker most used in the investigation of microeukaryotic communities in various contexts has been the gene that encodes the 18S rRNA (Pawlowski, 2014). In general, the primers used for sequencing specific regions of this gene produce good results. An example is the consistent use of these sequences and protocols in large projects such as BioMarKs/Biodiversa (Guillou et al., 2013), TARA Ocean Consortium (Pierella Karlusich et al., 2020), The Marine Microbial Eukaryote Transcriptome Sequencing Project (Keeling et al., 2014) and Earth Microbiome Project (Thompson et al., 2017). However unicellular eukaryotes, being very close phylogenetically to their hosts tend to be undersampled, with many of the reads obtained derived from the host animal (del Campo et al., 2019). Therefore, our reduced understanding of the Porifera microeukaryome may be a reflection of this methodological limitation. In the work of De Mares and collaborators (2017) even with the application of a deep sequencing methodology, about 85% of the reads belonged to the host. Also, in He and collaborators (2014) about 79% of all reads were from sponges and only 0.03% belonged to protists. This represents a huge data loss that has profound impact on our understanding of the microbiome of these animals. An alternative approach to diversity analysis is to use primers that only amplify the 18S rRNA gene of a specific group of protists of interest (Annenkova et al., 2011; Bass et al., 2015; Wecker et al., 2015; Hall et al., 2021). Another method consists of designing primers that do not amplify the copy of the gene of interest present in the host, something already applied for invertebrates such as insects (Waidele et al., 2017) and corals (Clerissi et al., 2018; Hume et al., 2018). For some one-off studies, such as ascertaining whether or not a particular group is present in sponge-derived samples, this may be a valid strategy. Although this procedure is not feasible for understanding biodiversity at the community level, it can be useful in the study of diseases caused by protists as a form of diagnosis, as is already widely used for several invertebrates (Gruebl et al., 2002; Liu et al., 2009; Pollock et al., 2011; Ríos et al., 2020).

Of the few more complete solutions, we can list three main strategies, each with its advantages and disadvantages (del Campo et al., 2020). The first one is to increase the sequencing depth in order to access the microeukaryotic fraction of the microbiome. However, this alternative is often not affordable for the vast majority of Porifera research laboratories given the still high costs of this process. Furthermore, as mentioned, deep sequencing, which can work very well for communities of prokaryotic organisms (Webster et al., 2010) can still be inefficient for protists (De Mares et al., 2017). The second option consists of using blocking primers for the host 18S rRNA gene. This method has already been successfully applied to investigate the microeukaryome of mammals (*Mammal_block_I-short_1391f* - https://earthmicrobiome.org/protocols-and-standards/18s/) and some lineages of marine invertebrates. One of the first characterizations of the microeukaryome of a holobiont was seen in the stony coral *Pocillopora damicornis* where Clerissi and collaborators (2018) designed blocking primers for Scleractinia that targeted variable loops of the host 18S rRNA gene and had a 3'-end spacer C3 CPG, preventing the elongation process during the polymerase chain reaction (Clerissi et al., 2018). The same approach was successfully applied to investigate protist diversity associated with the shrimp *Litopenaeus vannamei* (Liu et al., 2019) and the oyster *Crassostrea gigas* (Clerissi et al., 2020). However, to date it is unknown to us if there are works that used blocking primers in metabarcoding studies of Porifera-associated microeukaryotic communities. This gap in knowledge needs to be filled by creating primers, similar to those applied to other holobionts, with a 3'-end C3 spacer that prevent the amplification of the Porifera 18S rRNA gene at the phylum level or at the level of the four major classes: Demospongiae, Calcarea, Hexactinellida and Homoscleromorpha. The third option is the use of 'non-metazoan' primers (Bower et al. 2004, del Campo et al. 2019, 2020; Minardi et al., 2021), *i.e.*, primers that amplify sequences from protists without amplifying those of the animal host. Although these primers show promising numbers as they capture only 2.6% of metazoan sequences, about 74% of these sequences still belong to Porifera (del Campo et al., 2019). This shows that, at least for the study of the protist communities of the Porifera phylum, these primers still do not fully solve the host sequencing problem, but they configure one of the best options. Table 2 provides a summary of the main primers used in the investigation of the communities of unicellular eukaryotes associated with sponges as well as their percentage of 18S rRNA gene coverage. It is possible that the solution to this problem comes from the combination of these strategies: the use of deep sequencing and blocking primers of 18S rRNA gene from Porifera in addition to non-metazoan primers may be effective in reducing reads derived from the metazoan host (Fig. 3). The adoption of this approach will most likely reveal a previously unknown diversity and provide important insights into how the sponge functions as a holobiont.

5.2. The choice of the bioinformatics approach

With the rapid advancement of bioinformatics, we have seen the emergence of numerous computational tools. Many of them offer totally new resources, opening possibilities of biological data exploration unknown until now. Others come to correct flaws in older pipelines that, in face of new evidences, have lost scientific support for their use. Recently, these discussions gained strength within the context of bacterial and archaeal metabarcoding, mainly through the conceptual discussion between OTUs and ASVs (Amplicon Sequence Variants) (Callahan et al., 2017; Edgar, 2018; Prodan et al., 2020). Among these two concepts, OTUs were the first to be applied to microorganism sequence analysis. This approach is based on the idea of grouping sequences by relying on a reference library and using an arbitrary threshold, usually 97% similarity. In recent years, pipelines using an OTU-based approach have fallen into disuse given their difficulty in reproducibility and comparison with other studies (Callahan et al., 2017). In contrast, the use of ASVs as an atomic unit of analysis has been growing, and is even recommended for assessing protist diversity and community composition (Forster et al., 2019). ASVs, also called zOTUs (zero-radius OTU) or exact sequence variants, do not use the arbitrary 97% similarity threshold. Instead, ASV methods are based on the assumption that real biological sequences are more observed than erroneous sequences (Callahan et al., 2017; Edgar, 2018). Furthermore, the use of ASVs over OTUs is indicated since they allow for increased sensitivity when

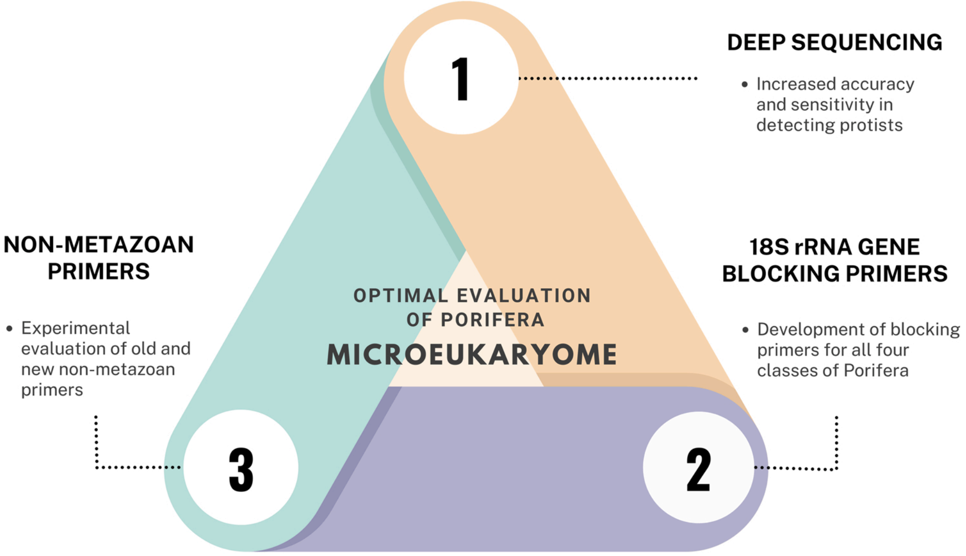

Fig. 3. Integrative proposal of the main metabarcoding strategies for optimal assessment of sponge-associated microeukaryotic communities.

investigating from community structures and patterns (Callahan et al., 2017; Needham et al., 2017; Forster et al., 2019). The ASVs allows much higher resolution than OTUs, generally being able to distinguish between different variants of the same species (Prodan et al., 2020). Furthermore, its use allows direct comparison between different studies, enabling new insights into the microbial community of interest.

To date, the few published studies that addressed the Porifera microeukaryome used pipelines that had OTUs as the end product. Using this procedure may introduce a new layer of underrepresentation of microeukaryotic diversity, since there is already the whole issue of 18S rRNA gene host similarity. By not being dependent on a reference database (closed-reference OTU analysis) or using an approach such as *de novo* OTU analysis (Callahan et al., 2017), ASVs studies can be compared between sponges from different locations in a more appropriate way. The adoption of more comprehensive, robust and reproducible pipelines such as USEARCH-UNOISE3 or DADA2 can be helpful in this regard (Prodan et al., 2020).

The researchers' preference for the metabarcoding approach to the study of Porifera microeukaryome goes beyond the cost-effectiveness of this technique. For a long time, it was one of the few valid strategies, since other approaches such as whole-metagenome shotgun sequencing, widely used for bacteria and archaea, presented difficult obstacles to overcome in microeukaryotes, such as the assembly of complex genomes (Keeling and del Campo, 2017; Massana and López-Escardó, 2022). However, with the progress of sequencing techniques, increase in computational capacity and the development of new bioinformatics tools and pipelines it is already possible to obtain metagenome-assembled genomes as demonstrated in the recent breakthrough by Delmont et al. (2022). Also, with novel methods such as EukDetect (Lind and Pollard, 2021), it is possible to identify microeukaryotes from metagenomic data in a similar manner accomplished by metabarcoding approaches. Therefore, with recent bioinformatic advances, the use of whole-metagenome shotgun sequencing may be an important strategy to identify the protists that are associated with sponges and understand their role in the holobiont.

6. Can the HMA/LMA dichotomy be applied to protists? And the specificity of the microeukaryome?

The terms *High Microbial Abundance* (HMA) and *Low Microbial Abundance* (LMA) were created to represent patterns of association between sponges and prokaryotes (Hentschel et al., 2003, 2006; Gloeckner et al., 2014). HMA-type sponges are defined as those that have around 10^8 to 10^{10} associated bacterial cells, while LMA have orders of magnitude to smaller around 10^5 to 10^6, closer to the concentrations found in seawater (Hentschel et al., 2006; Gloeckner et al., 2014). However, there still remain several questions that either urgently need answers or still require further evidence. One of them concerns the presence of this pattern in relation to the communities of protists and fungi associated with sponges, as raised by Hardoim and colleagues (2021). So far, we are unaware of investigations that seek to understand if it is possible to observe this same HMA and LMA pattern when it comes to unicellular eukaryotes.

Given the enormous morphological diversity of microeukaryotes, it is reasonable to consider that traditional classification methods such as transmission electron microscopy and fluorescent *in situ* hybridization cannot be applied efficiently in the same manner as is seen for Bacteria and Archaea (Hentschel et al., 2003; Bayer et al., 2014; Gloeckner et al., 2014). However, it would be interesting to investigate how sponges associated with protist groups with conspicuous morphological features such as diatoms and foraminifers (with their frustules and tests, respectively) are positioned within the HMA/LMA dichotomy. For this, it is expected that the progressive use of approaches such as the aforementioned combination of newer methods and strategies will increase the efficiency of metabarcoding studies (Fig. 3). As such, meta-studies that consider in their experimental design the diversity, community structure and function of the microeukaryome of various sponge species from different localities can better elucidate this question.

Another interesting question is to observe if the diversity is the same among the different groups of Porifera and if there is a specificity of this community. This investigative line has been seen in recent work such as the one comparing the microeukaryotic community of the LMA sponges *Dysidea avara* and *Dysidea etheria* and the HMA sponges *Aplysina aerophoba* and *Aplysina cauliformis* (De Mares et al., 2017). In this work the authors noted that the protist community found in these species failed to compose a sponge-specific cluster like the associated Bacteria and Archaea (De Mares et al., 2017). It is worth pointing out that the sponge-specific clusters and sponge-coral clusters with which the sequences of De Mares and collaborators were compared are based on only 95 eukaryotic sequences of 18S rRNA, most belonging to Metazoa and Fungi (Simister et al., 2012). Today the scientific community has access to a large number of eukaryotic sequences from both protists and fungi, which allows for a higher resolution of these clusters. On the other hand, the use of integrative approaches points to the ability of the host to regulate this microeukaryome by deliberately selecting symbionts (Hardoim et al., 2021). Added to this, through culture-dependent approaches, evidence is accumulating that there are sponge-enriched microeukaryotes in a similar way to what is observed for Bacteria and Archaea.

When considering this problematic in a physiological dimension, LMA sponges have less diversity of bacterial phyla related to important metabolic cycles such as carbon, nitrogen and phosphorus metabolism (Ribes et al., 2012; Gloeckner et al., 2014; Rix et al., 2020). Therefore, it would be interesting to investigate whether this need can be supplied by microeukaryotes that also possess such metabolizing capabilities. Also, genes related to polyketide synthases are often absent in LMA sponges, but widely distributed and diverse in HMA organisms (Hochmuth et al., 2010). Given the importance of these genes for the host primary and secondary metabolism (Fisch et al., 2009; Kaluzhnaya et al., 2021) it is possible that other microorganisms are producing these compounds, as may be the case of labyrinthulomycetes (Morabito et al., 2019), dinoflagellates and/or haptophytes (Kohli et al., 2016), which have polyketide synthases-like pathways. It would also be interesting to investigate if the microeukaryotic community functions redundantly or complementarily to the bacterial community associated with HMA sponges, producing or consuming compounds of interest to the holobiont along the lines of the work of Moitinho-Silva et al. (2017).

Assuming that this pattern exists for Porifera-associated protists, knowing which microeukaryotes make up the community of sponges classified as HMA or LMA may provide some valuable insights into holobiont interactions. We can hypothesize, for example, that LMA sponges may possess more predatory protists that can assist the sponge in keeping the population of associated bacteria, archaea, and viruses in check. Another hypothesis is that photosymbiont microeukaryotes such as "green algae" and diatoms can be found in greater abundance in HMA sponges, given the particularities of their body structure suited to host and support symbiotic microorganisms (Weisz et al., 2008).

7. Biotechnological applications of the Porifera microeukaryome

The interactions between humans and the organisms of the phylum Porifera are ancient, due to their use as bath sponges, medical material and other ends (Voultsiadou, 2007; Pronzato and Manconi, 2008). Only in the second half of the 20th century, did the use of sponges as sources of new molecules begin to be explored. Since then, these organisms have revealed a large number of compounds with a variety of pharmacological actions or unusual structures, being responsible for around 30% of all new molecules obtained from marine organisms (see Carroll et al., 2019 and other reviews of this series). At least part of this chemical richness is produced not by the sponge itself, but by its microbiota. Therefore, in recent years great attention has been paid to the biotechnological potential of bacteria and fungi, mostly for pharmacological uses. The variety of substances and actions are immense, from cytotoxic to antibiotics and antivirals (*e.g.* Thomas et al., 2010; Suryanarayanan, 2012; Vaca et al., 2013; Mehbub et al., 2016) including targeting the recent SARS-CoV2, responsible for the COVID-19 pandemic (Geahchan et al., 2021; Shady et al., 2021). In addition, there is an increase in the search for metabolic processes or compounds suitable for applied research, such as catalysts (de Oliveira et al., 2020b) or surfactants (Freitas-Silva et al., 2022). Considering that these components function optimally in the physiological conditions for the sponges - relatively mild temperatures, ionic concentrations and pH - the interest for the industry can be substantial.

On the other hand, as opposed to bacterial and fungal metabolism, it is reasonable to consider that possible applications of the sponge microeukaryome are still virtually unexplored. If one can draw a parallel with what has been given by other associate microorganisms which are already studied, such as bacteria, the potential is huge. Unicellular eukaryotes have diverse evolutionary histories, complex molecular mechanisms, and a significant capacity to synthesize bioactive molecules (Vallesi et al., 2020). Being an area that is still expanding within protistology itself, to date, natural product bioprospecting is focused on already well-known groups such as “green algae”, diatoms, ciliates and dinoflagellates (Vallesi et al., 2020). Many species from these taxa are closely associated with marine and freshwater sponges (Table 1). In this context, it is possible to highlight the sponge-associated dinoflagellates that produce okadaic acid, a potent phosphatase I and II inhibitor, (Tachibana et al., 1981; Müller et al., 2007) and some groups of unicellular “green algae” associated with *Plakortis halichondrioides* that can be a significant source of polyketides, a class of molecules that has several uses in biomedicine (Della Sala et al., 2014). Given the diversity of protists associated with Porifera and the fact that the association with the hosts provides a niche in which distinct strains and molecules can develop (Kita et al., 2010; Lopanik, 2014; Schorn et al., 2019), we can draw attention to some groups that may prove to be interesting sources of molecules and biotechnological processes.

Biotoxins produced by dinoflagellates and diatoms (also known as phycotoxins) that cause severe negative effects in humans can be potential targets in drug discovery pipelines (Alfonso et al., 2016; Vallesi et al., 2020). Potent metabolites such as gambierol (Cuypers et al., 2008), gymnodimine (Seki et al., 1995), yessotoxin (Alfonso et al., 2016) among others have been investigated as possible immunosuppressive, analgesic, antitumoral and with action capable of interrupting the progression of neurodegenerative and metabolic diseases (reviewed by Assunção et al., 2017). In addition, these protists and several species of "green algae" such as *Chaetoceros* sp., *Chlorella* sp. and *Nannochloropsis* sp. produce sulfated polysaccharides, molecules with various biological activities such as antimicrobial, anti-inflammatory and antioxidant besides the nutraceutical potential (Raposo et al., 2013).

Similar to bacteria and archaea, microeukaryotes also can be extremophiles and enzymes derived from microorganisms from harsh habitats are warranted in several segments of the industry (Coker, 2016). Ciliates as *Euplotes focardii* and diatoms of the genus *Fragilariopsis* (abundant in Antarctic sponges as seen in Bavestrello et al., 2000) that live in the Antarctic Sea have been the focus of several studies because they feature antifreeze proteins and enzymes that remain active at low temperatures (such as superoxide dismutases and cold-active α-amylase) (Bayer-Giraldi et al., 2010, 2020). Besides that, ciliates are a source of biomolecules and can be applied to processes of bioremediation, biotransformation and biocontrol as reviewed by Elguero et al. (2019).

Another taxon that deserves highlight is the Labyrinthulomycetes, a group of basal stramenopiles consistently isolated from sponge tissues (see Table 1). These microorganisms possess a large metabolic repertoire, which opens up the possibility of their use for a wide variety of biotechnological applications (Marchan et al., 2018). These protists are recognized for the synthesis of PUFAS, such as DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) as well as some species of diatoms and dinoflagellates (Marchan et al., 2018; Peltomaa et al., 2019). The significant production of these substances and ease of isolation from sponge tissues and other sources make these protists a target for exploitation by the nutraceutical industry, where they are used as a supplement for humans as well as in livestock and aquaculture (Marchan et al., 2018). These microeukaryotes possess in their metabolic repertoire the ability to produce carotenoids, squalene, and polysaccharide exudates. Carotenoids are natural pigments that can be used as ingredients in foods, cosmetics, and pharmaceuticals, as well as having antioxidant properties and being precursors to other bioactive molecules such as vitamins (Park et al., 2018). In turn, squalene is an essential hydrocarbon that acts as a precursor to steroids, bile acids, and vitamins, and also has antioxidant activities (Spanova and Daum, 2011). This compound is also widely used as a vaccine adjuvant, being an ingredient used in certain immunizers to promote a more robust immune response (Mendes et al., 2022). With one of the main sources of squalene being the liver of elasmobranchs such as deep-sea sharks (Spanova and Daum, 2011), populations of these animals become endangered being an unsustainable practice in the long term. In this context, Labyrinthulomycetes have been studied as possible alternatives to this problem (Aasen et al., 2016; Patel et al., 2020; Mendes et al., 2022). Polysaccharide exudates, on the other hand, also produced by these protists have several applications. Recently these compounds have been used for their antitumor and anticoagulant properties, but also their applications in the cosmetics industry (Nham Tran et al., 2020). Representatives of this group possess various enzymes capable of degrading substrates such as cellulose, lignin, starch, collagen, aromatic compounds, complex proteins, and lipids, also being used in biofuel production (Liu et al., 2014; Lee Chang et al., 2015; Li et al., 2021).

Further investigation into this biochemical profile of Porifera considering the portion of associated unicellular eukaryotes is therefore warranted. The use of omics tools can provide important information about the potentialities of the microeukaryome. However, this associated component can be only a tiny fraction of the whole sponge, and as such some previous enrichment is necessary. The initial isolation can be done either using *in vitro* sponge cell cultures of the host species or *bait* techniques, with antibiotics to eliminate the associate bacteria and fungi. Once in culture, these microorganisms may pose particular challenges, such as even lower density and slower growth than those observed for associated bacteria. Another valid strategy is to use fluorescence-activated cell sorting, a specialized type of flow cytometry, to obtain axenic cultures of the protists of interest, something that has already been applied to isolate dinoflagellates from marine plankton and to eliminate contaminations in microeukaryote cultures (Sinigalliano et al., 2009; Vu et al., 2018; Doppler et al., 2021). It is also important to note that often the interaction between the microorganism and its host is complex. Therefore, it is possible that metabolites of interest are not produced by the symbiont separately, requiring a more integrative approach to their isolation and culture (Schippers et al., 2012; Knobloch et al., 2019). In addition, if a strain is negative for one activity, it does not mean that it does not produce other molecules with different and interesting properties; therefore, it is recommendable to investigate multiple activities. In this sense, making the cultures available to other research groups for testing, with different equipment and techniques would also be highly desirable. The adaptation of pipelines developed for the bioprospection of fungi may be an interesting starting point for the discovery of molecules of biotechnological interest produced by protists associated with sponges.

8. Concluding remarks and future directions

We still have a long way to go before we understand the major roles that protists play in the biology of Porifera. However, we already have an outline of how these interactions occur. Culture-dependent approaches and direct observations remain essential for the in-depth study of these relationships, as it allows visualization of features that cannot be inferred from the genetic material, such as behavior and morphology. On the other hand, culture-independent analyses, based on omics, may help in understanding the influences of these microorganisms on sponge functioning as well as on the composition of this microeukaryotic community. We suggest that efforts related to the study of Porifera microeukaryotes focus in the following directions: (*i*) isolation and culture of protists from sponge tissues and integration with (*ii*) improved applications of metabarcoding approaches and whole-metagenome sequencing for inferences of community structure and function aiming at understanding the physiology and specificity of these associations; (*iii*) extensive meta-analyses to identify patterns of association along the lines of the HMA/LMA dichotomy and determine whether or not there is a specificity of these microeukaryotes; (*iv*) determine what are their contributions to biochemical profile of the sponges and how we can screen for potential biotechnological applications derived from these sponge-associated protists. Other questions that we can ask ourselves is about the extent of these protist-sponge relationships. How beneficial are they? In what context are they neutral? Can they become detrimental to one or both organisms involved? and when and under what conditions can this occur? Further topics worth mentioning are the interaction between the sponges and their associated protists concerning the particulate and dissolved organic matter. In summary, there are still many discoveries waiting to be made within the context of the much-neglected Porifera microeukaryome.

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CRediT authorship contribution statement

GN-S, CCPH and MRC contributed to the conceptualization of the review theme. GN-S and MRC contributed to the selection of appropriate literature. GN-S wrote the original draft and designed the figures. CCPH and MRC contributed to editing and reviewing the article. All authors approved the submitted version.

Declaration of competing interest

The authors declare no competing interests.

Data Availability

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

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