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Biofilms: An Extra Coat on Macroalgae

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<http://dx.doi.org/10.5772/63053>

Abstract

Biofilms are a mixture of complex communities of organisms mostly composed of diverse bacteria that vary depending on the surrounding environmental conditions induced by physical and chemical factors. In biofilms, symbionts play major roles in the relationship among organisms by the production of bioactive molecules involved in quorum sensing signaling. A cohesive structure of a multi-layer of extracellular polymeric substances (EPS) such as polysaccharides and proteins is the base of biofilm structural organization. Biofilms can be found in a variety of habitats, on free-living, on the surface of other organisms or inert surfaces, both in aquatic as well as terrestrial environments.

The importance of macroalgae in marine ecosystems is unquestionable. They are main key players along ocean coastlines, contributing to the overall primary production and providing shelter as well as food to many forms of life which can vary from microbes to large fish and mammals. Macroalgae are intimately associated with a huge microbial community coating their surface. As this microenvironment is very rich both in terms of biodiversity and food availability, life in it is very complex and competitive. The microorganisms, bacteria, archaea, fungi, microalgae like diatoms and protozoa, play fundamental roles in the development, defence and metabolism of the macroalgae. They benefit from the availability of diverse organic carbon sources commonly produced by the algae.

In this chapter, we intend to do a comprehensive revision of the actual state of the art of the biofilm community of macroalgae focusing on biodiversity, role played by both the microbiome and the host in this ecological system and its regulation namely through the quorum sensing. Furthermore, biofilm-related biotechnological applications, their role in macroalgae diseases and their influence in neighbor organisms will be also addressed.

Keywords: Macroalgae, Biofilm, Bacteria, Microalgae, quorum sensing

1. Introduction

Biofilms are complex, highly dynamic, structured ecosystems formed by a community of different microorganisms living attached to inert or living surfaces and embedded in a matrix designated extracellular polymeric substance (EPS). The colonization of a surface begins with EPS production by the initial bacterial colonizers through the formation of weak, reversible bonds called van der Waals forces and production of transparent exopolymer particles and their precursors by macroalgae that set the basis for the first bacterial colonizers settlement [1]. Several other microorganisms come after entering in this very competitive ecosystem where quorum sensing and antibiotic resistance are determinants in the development of the community. Observation of biofilms dates back to the seventeenth century when Antonie van Leeuwenhoek observed bacteria from the plaque biofilm of his teeth under his primitive microscope [2]. However, it was only in the 1940s that the concept of biofilm begun to arise with the works of Heukelekian and Heller [3] and of Zobell [4] whereas the first publication referring to the word biofilm appeared just in 1975 [5]. Due to the invisibility of microbial biofilms and inexistence of adequate methodologies for their study during many years, comprehension of this ecosystem is still scarce. The best studied biofilm systems are the ones associated with human body surfaces and technical surfaces like sensor heads or reverse osmosis membranes of desalination plants [6] and references therein. More recently, increasing attention has been paid to other systems like the epiphytic community on macroalgal surface. Several scientific groups are presently dedicated to the study of these biofilms with a consequent boost in the number of publications (see [7] for the increase in bacteria-macroalgae related publications). Since the description of the algal epiphyte bacterium, *Leucothrix mucor* [8, 9], more than 50 new bacterial genera and species have been described after their isolation from macroalgae [7]. This environment is proving to be prolific for the discovery of novel bacterial taxa. Several reviews on microbiome – macroalgae association have been done demonstrating the importance of microorganisms in this ecosystem [6, 7, 10–15]. The majority is mainly focused on the bacterial component of the microbiome which is the known main key player in these biofilms. Aspects of the importance of virus on macroalgal biofilms have recently appeared [16, 17].

The idea of considering biofilms as an extra “tissue” on the surface of eukaryotic organisms is based on the analogy between these two systems [6, 18]. We can thus consider the existence of an extra coat outside the macroalgal epidermis that gives an extra buffering between the host cells and the surrounding environment. In the biofilm, although cells are genetically different and variable, what does not happen in tissues, they interact functionally through nutritional exchange, communicate through quorum sensing and reproduce.

2. Macroalgal colonization and chemical interactions

Macroalgae inhabit an environment prone to epibiotic colonization (**Figure 1**) due to a constant pressure from the surrounding microbial community [19]. The relationship between bacteria in biofilm and their planktonic counterparts is of 1 to 2 orders of magnitude higher [6, 20]. However, Chan and McManus [21] in their study with *Polysiphonia lanosa* and *Ascomyllum*

nodosum found 100–10,000 times more bacteria associated with the algae than in the surrounding water. Bacteria on surfaces can reach densities higher than 10^7 cells cm^{-2} [22]. On the alga *Caulerpa racemosa* bacterial densities were of about 20×10^3 cells mm^{-2} and diatom densities of 40 cells mm^{-2} [23] while in *Ulva reticulata* there were 27×10^3 bacterial cells mm^{-2} and 5 diatom cells mm^{-2} [24]. On the kelp *Laminaria hyperborea* microbial cell densities varied between 8.3×10^2 cells cm^{-2} and 1.0×10^7 cells cm^{-2} [25] and on *Fucus vesiculosus*, Wahl et al. [26] reported values for epibacterial density of $7.7 \times 10^6 \pm 2.2 \times 10^6$ cells per cm^2 of algal thallus. In a study of the epiphytic diatom community on macroalgae from Iceland, Totti et al. [27] found diatom abundances between 7 ± 5 and 7524 ± 3491 cells mm^{-2} .

Figure 1. Macroalgal surface colonization of *Porphyra dioica* (A) and *Ulva* (B) by bacteria (A and B) and diatom (B) by light (A) and scanning electron (B) microscopy.

As perceived by these cell density numbers, marine macroalgae are one of the most important eukaryotes that provide excellent conditions for microbial colonization on their surfaces in the marine environment. Several factors are determinant for colonization. These include (1) the microtexturing (size and surface features) of the surface [28], (2) the production by the macroalgae of natural compounds with antifouling properties which include antimicrobials and quorum sensing disruptors [7, 26, 29–32], (3) the production of organic carbon compounds that trigger the chemotactic behavior of bacteria [33–34] and (4) the releasing of certain substrates that fulfill the nutritional needs of the epiphytic microbial community. On the other hand, macroalgae also benefit from the presence of this rich community as their growth and development are somehow dependent essentially on their bacteriome [14, 35].

In the microhabitat of the biofilm and on its interface with the macroalgae, complex chemical interactions occur. Both basibiont (macroalgae) and epibiont (microorganisms in the biofilm) contribute to this myriad of compounds. The macroalgae supply bacteria with oxygen and fixed carbon which is released as extracellular exopolysaccharides such as alginate, cellulose and mannitol [36–38]. Bacteria, through the mineralization of organic compounds released in the biofilm, supply the macroalgae with CO_2 , minerals, vitamins and growth factors [39–42]. In the mineralization process, many other molecules are formed which enrich the biofilm microenvironment and contribute to its biomass formation.

Growth factors produced by bacteria are phytohormones and biostimulators of growth and development [7]. Strains isolated from *Ulva mutabilis*, *Roseobacter*, *Sulfitobacter*, and *Halomonas*, associated with strain *Cytophaga* were effective in the development of the *Ulva* gametes into normal thalli due to specific regulator factors (cytokinin-type and auxin-type, respectively) excreted into the environment [43]. Secondary metabolites produced by bacteria in the biofilm are also fundamental for the completion of macroalgal life cycle and spore release and germination. Mixed microbial biofilms were shown to stimulate the rate of settlement of zoospores of the green alga *Enteromorpha* [44] and a positive correlation was observed between the number of zoospores settling and the number of bacteria in the biofilm [45]. Strains of *Vibrio* and *Shewanella* showed stimulation of spore settlement while *Pseudoalteromonas* strains inhibited settlement and also induced paralysis and lysing of *Enteromorpha* zoospores [46]. A similar stimulatory effect was also observed in the zoospore settlement of *Ulva linza* [47]. *Ulva* zoospores have the capacity to sense a range of different bacteria produced N-acyl homoserine lactone (AHL) molecules which interfere with their settlement [48].

Bacteria are fundamental for the growth and morphogenesis of several macroalgae. Provasoli [49] observed that *Ulva* in axenic culture did not develop normal morphology which was recovered after inoculation of bacteria previously isolated from this macroalgae [50]. Similarly, other Chlorophyta, *Monostroma oxyspermu* and *U. linza* that also lose their normal morphology when in axenic cultures could reestablished their normal morphology after culture incubation with bacterial extracts or inoculation with an appropriate bacterial community [47, 51]. Bacteria with morphogenesis-inducing activity were identified to be related to the genera *Flavobacterium*, *Vibrio*, *Pseudomonas*, *Deleya*, *Escherichia* and Gram-positive cocci [52]. Matsuo et al. [53] identified 40 active strains that were affiliated to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex, mainly in a clade comprising *Zobellia uliginosa*. In 2005, Matsuo et al. [54] proved that it was the specific bacterial strain YM2 that produced a secondary metabolite designated thallusin that was responsible for the normal development of ulcacean foliose. But this interaction is not restricted to Chlorophyta. The Rhodophyta *Pyropia yezoensis* (former name "*Porphyra yezoensis*") also needs bacteria to induce normal morphogenesis in its gametophytic phase [55]. Recently, Fukui et al. [56] identified the bacteria that induced normal morphogenesis in this red alga. They are members of *Alfaproteobacteria*, *Gammaproteobacteria* and *Flavobacteri* with special relevance to strains of *Hyphomonas*. Bacteria are, thus, fundamental for algal morphogenesis and life cycle development.

Furthermore, bacteria are also sources of fixed nitrogen and detoxifying compounds [7, 57, 58]. Nitrogen-fixing cyanobacteria are known to provide fixed nitrogen to macroalgae. These include *Calothrix* sp., *Anabaena* sp., and *Phormidium* sp. on *Codium* species [59, 60], *Dichothrix fucicola* on *Sargassum natans* and *Sargassum fluitans* [61, 62] and *Azotobacter* sp. on *Codium fragile* [63].

Another advantage of microbial community in macroalgal biofilms is their ability of scavenging of heavy metal [42, 58] or crude oil [64]. Many bacteria also play a fundamental role in biotransformation and nutrient cycling in the oceans due to the capacity to decompose the macroalgal cell walls [7, 65]. Bacteria, with the appropriate enzymatic machinery, contribute to the decay process of seaweeds [66].

Macroalgal epiphytic colonization is very uneven [67]. Macroalgae inhabiting the same environment or closely related species like *F. vesiculosus* and *Fucus evanescens* can possess very different levels of fouling [68]. These can be justified by different levels of antifouling defence mechanisms. Macroalgae defend themselves from invaders through the mechanical sloughing off of the outermost cell layer [7, 69] and the release of antimicrobials including reactive species of oxygen [7, 70] and bacterial communication blockers, the furanones [71]. Quorum sensing inhibitors and antimicrobial compounds produced by the bacteria are fundamental in the protection against pathogens, herbivores and fouling. These act in conjunction with the compounds produced by the macroalgae.

Macroalgae are a rich source of bioactive compounds against colonizing organisms. They are assisted on this task by the many antimicrobials produced by microorganisms on their biofilms, production that is widespread among bacteria [72–75]. Members of the genera *Pseudomonas*, *Pseudoalteromonas*, *Stenotrophomonas*, *Vibrio*, *Aeromonas*, *Shewanella*, *Streptomyces* and *Bacillus* are common antimicrobial producers from macroalgae [75]. Goecke et al. [7] provide several examples of antimicrobials produced by bacteria associated with macroalgae.

The oxidative burst response is based on the production of large amounts of reactive oxygen species by the macroalgae inducing the death of undesired microorganisms like pathogens and also controlling bacterial growth on algal surfaces. Elicitors of oxidative burst, signals that mediate the activation of cell-based induced defence responses, were recognized to be glycoproteins and glycopeptides, low-molecular weight peptides in the red agarophyte *Gracilaria conferta* [76] and oligomeric degradation products of alginate and bacterial lipopolysaccharides (LPS) in the brown algal kelp *Laminaria digitata* [77, 78]. Reaction to alginate oligosaccharides was also observed in other Laminariales [79]. Furthermore, in *L. digitata* arachidonic acid, linolenic acid and methyl jasmonate were found to be strong triggers of an oxidative burst [80].

Bacteria, once thought to be silent, were discovered to have specific intra- and inter-species signaling mechanism of communication that has been named quorum sensing (QS). They communicate via production of chemical signals with multifunctional activity due to their interacting QS gene regulatory ‘modules’ which are able to produce several different molecules, from the same or different chemical class that interact in hierarchies [81–83]. These molecules act as gene regulators of the population behavior in food uptake or common defence or escape when the survival of the community is at risk [81, 84]. In addition to communication with other microbes, bacteria also perceive molecules from eukaryotes that are known to be key factors in host-epibiont interaction [15, 83].

QS communication was discovered in the 1990s and proved to fulfill different ecological purposes like the induction of biofilm formation, movement of bacteria and the production of bioluminescence, antibiotic and virulence factors [85–87]. Similar to the QS signals that balance the equilibrium of the community, quorum quenching (QQ) signals are inhibitors of QS and also have impact on biofilm communities.

Halogenated furanones are structural analogues to N-acyl homoserine lactones (AHLs) and interfere with AHL-regulated processes and impair biofilm formation [88–90]. The first

compound with QS disruptor capacity isolated from a marine source, the red alga *Delisea pulchra*, was furanone. It is used by this alga to control surface colonization of marine bacteria [91]. *D. pulchra* has been used as a model organism for understanding the ecological role of secondary metabolites as natural antifoulants [71]. Furanones are produced by the macroalgae and on their surfaces at a concentration where they regulate bacterial colonization and the settlement of epibiota by interfering with the acylated homoserine lactone regulatory system (quorum-sensing pathway) in Gram-negative bacteria and with the alternative AI-2 signaling system in Gram-negative and Gram-positive bacteria [71, 83]. Furthermore, they also interfere with the attack mode of action of bacteria by inhibiting the expression of bacterial exo-enzymes that actively degrade components of the immune system thereby enhancing macroalgal immune response.

Since the discovery of these QS inhibitors, other studies lead to the isolation of more compounds able to block QS signal like the mixture of floridoside, betonicine and isethionic acid, isolated from the red alga *Ahnfeltiopsis flabelliformis*, that inhibited the activity of N-octanoyl-DL-homoserine lactone [92]. Moreover, studies using several macroalgae revealed a strong QS inhibitor produced by *Asparagopsis taxiformis* [93].

The production of QS signals not only affects bacterial responses but also the settlement of the green macroalga *Ulva* that react to AHL signaling of *Vibrio anguillarum* in the selection of surface sites for zoospores attachment [48]. Moreover, the study of the epi- and endobiont bacterial community associated to the macroalgae *Ulva fasciata*, *Ulva lactuca*, *Gracilaria corticata* and *Gracilaria dura* allowed the isolation of several strains of which some Gram-negative strains were able to induce carpospore liberation from *G. dura* by AHLs production.

AHLs also have effect on diatoms in the biofilm matrix as was observed by Yang et al. [94]. 3,4-dibromo-2(5)H-furanone, 4-nitropyridine-N-oxide and indole were able to decrease significantly the growth of two marine diatoms, *Cylindrotheca* sp. and *Nitzschia closterium*.

Although biofilms are important for macroalgae biology, they can also bring on detrimental effects as their members compete for nutrients, interfere with gaseous exchange, form a barrier to light that is fundamental for macroalgal photosynthesis and can lead to disease and degradation of algal tissue [95–97].

Similar to humans and plants, macroalgae possess their own defence mechanisms and immunity adaptations developed to survive and coexist with pathogenic or phycocolloids degrader's organisms [18, 98]. It is suggested that, since microbes are more predominant in water than in air, macroalgae evolved to more resistant forms by elimination of more susceptible individuals and resistance of the ones capable of producing chemicals for self-defence [99, 100]. Actually, few are the reports on the study of pathogenic microorganisms in macroalgae. Although, studies on algal diseases have risen due to the increase of the use of algae in food industry, seaweed mariculture and to the consequences of global warming and its impact on ocean temperature [96, 101]. The most studied pathogen-macroalgae systems studied are the host-specificity infection by *Roseobacter* in *Prionitis lanceolata*, that induces the formation of a tumor-like growth in the thallus [102] and the induction of bleaching in the red alga *Delisea pulchra* by *Nautella italica* R11 and *Phaeobacter* sp.LSS9 [16, 101, 103, 104]. Also, studies on

Laminaria religiosa health revealed that *Alteromonas* sp. is a pathogenic strain that allied with abiotic factors induce severe damage and bleaching to the alga [105]. Not only bacteria and fungi threaten macroalgae, but also the pathogenic epiphytic alga *Neosiphonia apiculate* induces reduction of carrageenan production and secondary bacterial infection [106]. This constant fight to survive invasions through the production of chemical compounds like antibacterials led to the isolation of several compounds from marine macroalgae, such as lobophorolide isolated from *Lobophora variegata* with activity against pathogenic and saprophytic fungi [99].

The biofilm community present on macroalgae not only has influence on the host life but also on other eukaryotes living nearby. Effects on sea urchin larval settlement by coralline algae biofilm communities [107] and on *Mytilus edulis* larval settlement by producing attraction or repellent signals to mussels [108] have been observed. These studies reveal the impact of the macroalgae biofilm in the surrounding organisms.

3. The diversity of microbial community on macroalgae

In marine environments, biofilms are mainly formed by bacteria but also by several different eukaryotes such as diatoms, fungi and protozoa [14] in a ratio of 640:4:1 of Bacteria:Diatoms:Flagellates [22].

3.1. Bacterial diversity

Hollants and collaborators in 2013 [12] did an exhaustive analysis of the macroalgae-bacterial diversity compiling information from 55 years and 159 seaweed species (36 green, 72 red, and 51 brown marine macroalgae). They concluded that bacteria associated to macroalgae belong to the phyla *Proteobacteria*, *Bacteroidetes* (CFB group), *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Fusobacteria*, *Tenericutes*, and the candidate division OP11. The dominant groups were *Gammaproteobacteria* with 37% relative abundance in published records, followed by the CFB group (20%), *Alphaproteobacteria* (13%), *Firmicutes* (10%), and *Actinobacteria* (9%). At the order level, *Flavobacteriales* (14%), *Alteromonadales* (12%), *Vibrionales* (10%), *Pseudomonadales* (9%), *Bacillales* (9%), *Actinomycetales* (8%), and *Rhodobacterales* (7%) were the most abundant. On their analysis they also found that all groups have been isolated from the three lineages of macroalgae, Chlorophyta, Rhodophyta and Heterokontophyta (Phaeophyceae) but differences were observed between them: *Bacteroidetes* and *Alphaproteobacteria* were more associated to green macroalgae while species of *Firmicutes*, *Actinobacteria*, and *Planctomycetes* to red and brown algae. On their study at lower taxonomic level (genus and species), bacterial taxa variability was found in closely related seaweeds. Genera like *Alteromonas*, *Bacillus*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas*, and *Vibrio*, in a total of 33 genera, were frequently associated with the three groups of macroalgae while *Cytophaga*, *Planococcus* and *Tenacibaculum* appear commonly in green and red but not in brown seaweeds.

During many years, the study of macroalgal biofilm diversity was based on organism isolation in pure cultures with their subsequent taxonomic characterization. With the development of

molecular and new generation sequencing techniques, a much more precise and detailed assessment of diversity has been possible. Presently, our knowledge, essentially at bacterial level, has been extended to many groups of organisms known for their difficulty to be brought into culture.

Longford et al. [109], using 16S rRNA gene library analysis, compared the bacterial community of the red macroalga *Delisea pulchra* and the green intertidal alga *Ulva australis*. *D. pulchra* contained 7 phyla and *U. australis* only 4 phyla and the two shared representatives from *Alpha*-, *Delta*- and *Gammaproteobacteria*, *Planctomycetes* and *Bacteroidetes*. Alpha diversity was relatively high in *D. pulchra* and comparatively lower in *U. australis*. Beta diversity at the species level, the measurement of the turnover of species between two sites in terms of gain or loss of species, was high as no species showed universal distribution between the two macroalgae.

Tujula et al. [110] in a catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and denaturing gradient gel electrophoresis (DGGE) study found that the epiphytic microbial community of *U. australis* was mainly constituted by bacteria (90%) of which 70% were *Alphaproteobacteria* (mainly the *Roseobacter* clade) and 13% *Bacteroidetes*. The 16S rRNA gene clone libraries of *Ulva australis* showed that its biofilm was dominated by bacterial members of *Alphaproteobacteria* and *Bacteroidetes*, especially within the *Rhodobacteriaceae*, *Sphingomonadaceae*, *Flavobacteriaceae* and *Saprospiraceae* families [111]. No consistent species-specific bacterial community was observed between libraries.

The brown alga *Laminaria saccharina*, now classified as *Saccharina latissima*, was studied by Staufenberger et al. [112]. Its bacterial community, as revealed by DGGE and 16S rRNA gene clone libraries, varied in the different parts of the alga (rhizoid, cauloid, meristem and phylloid) and the bacterial phylotypes obtained were affiliated to *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* groups.

The epiphytic bacteria on the macroalga *Chara aspera* was colonized mostly by members of the *Cytophaga-Flavobacteria-Bacteroidetes* group but also by *Betaproteobacteria*, *Gammaproteobacteria*, *Planctomycetes* and *Actinomycetes* [113].

Hengst et al. [114] studied the composition and structure of bacterial communities on three macroalgae from two coastal areas in the Northern Chile varying in copper concentration in seawater. They found that the bacterial communities' structure was determined by the algal host and time dependent. Significant changes in the bacterial community structure induced by copper were observed in *Ulva* spp. but not in *Scytosiphon lomentaria* and *Lessonia nigrescens*. The phyla encountered in the algal biofilm were *Bacteroidetes*, *Alphaproteobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Cyanobacteria*. *Verrucomicrobia* were exclusively found in polluted sites. The bacterial communities in this study were determined by algal species > temporal changes > copper levels.

In a DGGE and clone libraries study, Lachnit et al. [115] verified that the macroalgae *F. vesiculosus* (brown), *Gracilaria vermiculophylla* (red) and *Ulva intestinalis* (green) living in close proximity showed consistent seasonal differences in their bacterial community at phylum level. However, each macroalgal species possessed a species-specific and temporally adapted epiphytic bacterial community. *F. vesiculosus* harbored *Alphaproteobacteria*, *Bacteroidetes*,

Verrucomicrobia and *Cyanobacteria* in summer while in winter *Cyanobacteria* were not observed and the abundance of *Gammaproteobacteria* increased. In summer, *G. vermiculophylla* possessed mainly *Alphaproteobacteria* and *Bacteroidetes* and in winter the phylum *Deinococcus* was detected. In *U. intestinalis*, *Alphaproteobacteria* was the major phylum both in winter and summer but also *Gammaproteobacteria* and *Bacteroidetes* were present. Phyla also detected in this study were *Beta*-, *Epsilon*- and *Deltaproteobacteria*, *Planctomycetes*, *Actinobacteria* and OD1-OP11-WS6-TM7. *Octadecabacter arcticus*, *Granulosicoccus antarcticus*, a *Bacteroidetes* strain (EU246795), *Roseibacillus* spp. and *Planctomyces* sp. (EF591887) were the closest related bacterial strains to the operational taxonomic units (OTUs) found on *F. vesiculosus* while *Mesorhizobium* (DQ269119), *Hyphomonadaceae* (EU642858), *Actinobacterium* (DQ289932), *Bacteroidetes* (DQ269100), *Roseobacter* (AY167339), *Cytophaga* (AB015265) and *Bacteroidetes* (DQ269042) were the ones observed in *G. vermiculophylla*.

The bacteriome of the kelp *L. hyperborea* from two sites on the southwestern coast of Norway was studied by DGGE by Bengtsson et al. [25]. They found that *Planctomycetes* and *Alphaproteobacteria* were the most frequent phyla but also *Verrucomicrobia*, *Cyanobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Bacteroidetes* were detected throughout the year.

The macroalgae *Osmundaria volubilis*, *Phyllophora crispa*, and *Laminaria rodriguezii*, from the Balearic Islands (western Mediterranean Sea), were found to have their surfaces dominated by bacterial ammonium monooxygenase (*amoA*) genes as determined by quantitative PCR analyses [116]. Comparatively lower levels were found for archaeal counterparts. The ammonium monooxygenase bacteria (AOB) community (15 operational taxonomic units (OTUs)) was mainly composed of members of *Nitrosospirra* spp. and of *Nitrosomonas europaea* and the ammonia-oxidizing archaea (AOA, 43 OTUs) showed higher diversity. Trias et al. [116] estimated 6 times higher abundance of AOB comparatively to AOA and that the former accounted for about 1% of the total bacterial community on the algal surfaces.

The effect of temperature on the bacteriome of the brown macroalga *F. vesiculosus* was studied by DGGE and 454 pyrosequencing of the 16S rRNA gene [117]. Of the 21 present phyla, the dominant OUTs found were *Proteobacteria* (~68%) and *Bacteroidetes* (~18%). *Alphaproteobacteria* and *Rhodobacteriaceae* were respectively the prevalent class and family in all the temperatures but this family more than doubled in abundance from the lowest to the highest temperature assayed. Temperature did not influence cell density but was responsible for 20% of the variation in the bacterial community composition. Furthermore, Stratil et al. [118] also analyzed, by 454 pyrosequencing of 16S rRNA gene sequences, the effect of salinity on the biofilm of *F. vesiculosus* and observed a significant influence of salinity on bacterial OTU richness and evenness. Alpha diversity, the number of species and the proportion in which each species is represented in the community, was lower at the lowest salinity assayed (5 ‰), in which the more relevant bacterial group was *Betaproteobacteria*. Members of this phylum were absent at the two higher salinities assayed (19 and 25 ‰) where *Gammaproteobacteria* strains dominated. Compared to the colonization of a non-living substrate (stone), *F. vesiculosus* was less colonized by *Cyanobacteria* and microalgal chloroplasts (probably diatoms) revealing antifouling ability against these organisms. Stratil et al. [118] results showed the importance of salinity in the structuring of algal biofilms.

Martin et al. [119] showed that *A. nodosum* biofilm is significantly enriched in macroalgal-polysaccharide-degrading bacteria. Of the cultivable bacterial subpopulation associated with *A. nodosum*, about 25% were algal polysaccharide degraders [119] which belonged to the classes *Flavobacteria* (*Cellulophaga*, *Maribacter*, *Algibacter*, and *Zobellia*) and *Gammaproteobacteria* (*Pseudoalteromonas*, *Vibrio*, *Cobetia*, *Shewanella*, *Colwellia*, *Marinomonas*, and *Paraglaceciola*). Regarding the total bacterial isolates obtained, the most abundant groups observed were *Bacteroidetes* and *Gammaproteobacteria*. However, phyla like *Planctomycetes* (known to possess enzymatic machinery for macroalgae polysaccharides degradation) and *Cyanobacteria* commonly found on brown algae were not isolated.

Using 16S rRNA gene clone libraries, Wu et al. [120] observed a host-specific but temporally and spatially variable epibacterial community on the surface of the four red macroalgae, *Gracilaria lemaneiformis*, *Gloiopeltis furcata*, *Mazzaella* sp. and *Porphyra yezoensis*. *Alfa-* and *Gammaproteobacteria* and *Bacteroidetes* dominated these communities but *Deinococcus-Thermus*, *Spirochaetes* and *Epsilonproteobacteria* were also found. The most frequent genera in the four clone libraries were *Pseudoalteromonas* in *Gr. lemaneiformis* and *Gl. furcata*, *Sulfitobacter* in *P. yezoensis* and an undefined cluster within *Deinococcus-Thermus* in *Mazzaella* sp.

The composition of *Porphyra umbilicalis* bacterial community was analyzed by high-throughput pyrosequencing and classified into eight phyla: *Bacteroidetes*, *Proteobacteria*, *Planctomycetes*, *Chloroflexi*, *Actinobacteria*, *Deinococcus-Thermus*, *Firmicutes*, and the candidate division TM7 [121]. In the *Bacteroidetes*, *Sphingobacteria* was the most represented group. A core microbiome which included *Granulosicoccus* (*Gammaproteobacteria*), *Hyphomonadaceae*, *Hellea* and *Loktanella* (*Alphaproteobacteria*), *Iamia* (*Actinobacteria*), members of the *Sphingobacteria* (namely *Aureispira*, *Haliscomenobacter*, *Lewinella*, *Saprospiraceae* and *Chitinophagaceae*), *Tenacibaculum* (*Flavobacteria*) and *Rhodopirellula* (*Planctomycetes*) was present in *P. umbilicalis*. Richer and more diverse bacterial communities were observed in algae from autumn than in the ones from winter.

Previous studies have shown that planctomycetes are common inhabitants in macroalgal biofilms [111, 115, 122]. In 2014, Bondoso et al. [123] analyzed the *Planctomycetes* communities epiphytic on six different macroalgae (red - *Chondrus crispus*, *Mastocarpus stellatus*, *Porphyra dioica*; brown – *Fucus spiralis*, *Sargassum muticum*; and green – *Ulva* sp.) from two rocky beaches in the North of Portugal. Based on DGGE profiles, the lowest diversity was observed in *F. spiralis* and the highest in *M. stellatus* and *P. dioica* from Porto and each alga revealed a planctomycetes specific community.

In the various studies of bacterial diversity associated to macroalgae, several patterns were observed. Seasonal and geographical (Baltic Sea and North Sea) differences in bacterial communities of *Saccharina latissima* were observed by Staufenberg et al. [112]. Tujula et al. [110] also observed differences between *U. australis* individuals between seasons and from both the same and different tidal pools. This difference was highest in winter. However, they found that there was a constant sub-population present (members of the *Alphaproteobacteria* and the *Bacteroidetes*). On a comparative DGGE study of six macroalgae (*Fucus serratus*, *F. vesiculosus*, *L. saccharina*, *Ulva compressa*, *Delesseria sanguinea* and *Phycodrys rubens*) from the Baltic and North Seas, Lachnit et al. [124] observed the existence of significant differences

between the epibacterial communities of these algae that differed less between regions than between host species and were more similar on closely related host species. This work suggested that the biofilm communities are controlled by the macroalgae. Lachnit et al. [115] also verified that the macroalgae *F. vesiculosus*, *G. vermiculophylla* and *U. intestinalis* living in close proximity showed seasonal differences in their bacterial community at phylum level which were seasonally consistent. However, each macroalgal species possessed a species-specific and temporally adapted epiphytic bacterial community.

Although changes occur over season, life span and macroalgal thallus parts, specific association seems to exist between bacteria and macroalgae [25, 110, 112]. However, Burke et al. [111] verified that *Ulva australis* individuals co-inhabiting a common environment harbored a unique assemblage of bacterial species and that this community was established based on functional genes and not on the taxonomy of the species [125].

3.2. Viriome studies

Recently, attention started to be paid to the viruses associated with macroalgae [17]. The virome associated with the red macroalga, *Delisea pulchra* was analyzed and virus-like particles were icosahedral, bacilliform to coiled pleomorphic and bacteriophages. The viruses found suggest an infection role as dsRNA viruses were affiliated to the genus Totivirus and a ssRNA to the order Picornavirales, both known to infect, respectively, plant pathogenic fungi and marine diatoms.

3.3. Fungal diversity

Singh et al. [126] did a comprehensive revision on marine fungi associated with the three groups of seaweeds. Their relationship towards the macroalgae can be of parasitism, saprotrophic nature or symbiosis, being pathogens and parasites the dominant ones [127]. Many macroalgae of the three lineages can harbor a diverse assemblage of marine fungi. Endosymbiotic representatives are members of the genera *Acremonium*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Geomyces*, *Penicillium*, and *Phoma* of which Ascomycota and anamorphic fungi are the most common [126]. *Geomyces* species, *Penicillium* sp. and *Metschnikowia australis* were the most common fungi associated with eight macroalgae from Antarctica [128]. Chlorophyta seems to be the macroalgae more densely colonized by fungi but with a lower diversity and Pheophyceae revealed the highest diversity [129]. The marine fungus *Pestalotia* sp. was isolated from the surface of the brown alga *Rosenvingea* sp. [130]. Zhang et al. [131] studied the fungal community associated with four species of red alga, two species of brown alga and two species of green alga, and verified that the brown alga *Sargassum thunbergii*, and the red alga *G. lemaneiformis* yielded many more cultivable isolates than the other ones (*Rhodomela confervoides*, *Gelidium amansii*, *A. flabelliformis*, *Colpomenia sinuosa*, *Enteromorpha prolifera* and *Ulva pertusa*). *Penicillium glabrum*, *Fusarium oxysporum*, and *Alternaria alternata* were also identified in this study.

Porphyra red rot disease caused by *Pythium porphyrae* has important economic impact in countries like Japan and China where this alga is intensively cultivated. Li et al. [132] studied

oomycetes and fungi parasites of marine macroalgae and they found a total of 13 species that are parasites being some obligate pathogens (*Eurychasma dicksonii*, *Eurychasmidium tumefaciens*, *Olpidiopsis porphyrae*, *Petersenia lobata*, *Petersenia palmariae*, *Petersenia pollagaster*, *Pontisma antithamnionis*, *Pontisma feldmannii*, *Pontisma lagenidioides*, *Pythium marinum*, *Pythium porphyrae*, *Sirolopidium andreei* and *Sirolopidium bryopsidis*).

Using 28S rRNA gene PCR-DGGE and real-time PCR analyses, Zuccaro et al. [133] studied the filamentous fungi present in healthy and decaying *Fucus serratus* thalli. They found *Lindra*, *Lulworthia*, *Engyodontium*, *Sigmoidea/Corollospora* complex, and *Emericellopsis/Acremonium*-like ribotypes. By cultivation approach, *Sigmoidea marina* was the fungus highly isolated. In decaying thalli, the fungal community changed and was composed of members of the Dothideomycetes.

3.4. Algal diversity

The epiphytic microalgal community on macroalgae has been studied by a restricted number of authors [27, 31, 134–138]. This community is mostly dominated by benthic diatoms and some few centric species possessing an attached mode of life. The composition of benthic diatoms on macroalgal biofilms can be modulated by several environmental conditions including nutrients, salinity, light conditions and hydrodynamic regime as well as by biological factors like grazing, adhesive capacity of diatoms and chemical interactions with the host [27] and references therein.

In a study performed by Al-Handal and Wulff [134], of the 50 epiphytic diatoms identified, *Cocconeis* spp., *Entopyla australis* var. *gigantea*, *Grammatophora arctica*, *Licmophora Antarctica* and *Pseudogomphonema kamtschaticum* were the most common taxa detected on the surface of several macroalgae which showed a different behavior as host: Chlorophyta harbored no diatoms; Phaeophytes an higher number; and Rhodophyta species, *Pantoneura plocamioides*, *Delesseria lancifolia* and *Georgiella confluens* were the most colonized macroalgae.

Based on molecular data and/or SEM characteristics, two abundant diatoms epiphytic on the assimilation hairs of the brown macroalga *Chordaria flagelliformis* were identified as *Fragilaria barbararum* and *Fragilaria striatula* [135].

Three macroalgae, the brown alga *Pilayella littoralis*, the red alga *Ceramium gobii*, and the green alga *Cladophora glomerata* were comparatively analyzed regarding their diatom colonization [136]. This was higher in spring and in higher salinity (Baltic Sea comparatively to Bothnian Sea). The green alga harbors lower diatom numbers. Although the brown macroalgae was the one possessing the highest diatom numbers, it presented the lowest community diversity. The highest community diversity was found on *Ceramium*.

Diatoms epiphytic on red macroalgae living under the sea ice showed a species pattern with depth in which *Cocconeis fasciolata* dominated at 10 and 15 m, *Porosira glacialis* at 20 m and *Eunotogramma marginopunctatum* at 25 m [137].

Totti et al. [27] in their study of the epiphytic communities on macroalgae from Iceland detected that erect growth forms of diatoms represented 50% of its community (*Achnanthes* cf. *bre-*

vipes var. *parvula*, *Tabularia investiens*, *T. fasciculata*, *Hyalosira* cf. *delicatula*, *Gomphoseptatum aestuarii*, *Pseudogomphonema plinskii*), adenate diatoms 29% (*Cocconeis stauroneiformis*, *C. scutellum*) and motile forms 21% (*Nitzschia* cf. *amphibia* and *Navicula perminuta*).

Tanaka [139] studied the adhesive capacity of diatoms and verified no close correlation with cell size, their cell form, motility, and mucus secretion. Also, no macroalgal species specificity existed in diatom colonization which was composed preferentially by *Navicula* sp., *Cocconeis* spp., *Gornphonema* sp., *Nitzschia closterium* and *Synedra tabulate*.

4. Biotechnological potential of macroalgae biofilms

The identification of thousands of microbial species and the increase in knowledge on macroalgal biofilms diversity and functioning lead to the valorization of its diversity with the development of several products in a wide variety of fields. The communities living on the surface of macroalgae benefit from a mutualist relationship with their host. The macroalgae are a reliable source of nutrients and on the other hand epiphytic bacteria and fungi help their hosts by producing bioactive molecules that protect all the community from unwanted invaders [39]. The microorganisms in a biofilm community compete against each other and protect themselves from other surrounding pelagic microorganisms by working together as a team producing different kinds of chemicals such as antifungal, antiprotozoal, anti-settlement and antibiotic molecules [39, 74, 126, 140]. It is the high competition in these communities that induce microorganisms to produce allelochemicals that can be applied in industries such as pharmaceuticals, cosmetic industry or even in agriculture [126].

Globally, hundreds of new natural molecules produced by marine organisms are discovered in a temporal pattern wherein the last discovery peak of new metabolites happened 10 years ago [141]. These authors tried to explain this effect with the need of improvement of the techniques used to discover new compounds. The genomic data now available, the high-throughput assays for cytotoxicity in cell-based screening and the automation in nuclear magnetic resonance (NMR) and mass spectroscopy assist the discovery, the accurate identification and validation of new leads to treat human diseases [141]. This review emphasizes the importance of the searching of new compounds from macroalgae and their associated microorganisms. The highest number of bioactive hits found was provided from marine invertebrates, although in the last decades it was discovered that the compounds were actually produced by the associated/symbiotic microorganisms. Even though marine microorganisms provided the highest percentages of bioactive compounds, microorganisms associated to algae are still a minority [141].

Nowadays, the boom of reports that address the search of bioactive compounds produced by macroalgae-associated microorganisms reflects the importance and novelty of the compounds obtained from these sources. Singh et al. [126] described in a review several reports regarding antimicrobial compounds from seaweeds-associated bacteria and fungi published until 2014. Furthermore, they provided details on the bacteria and fungi associated with macroalgae that are producers of bioactive molecules. It is worth mentioning the ecological role of several new

compounds such as haliangicin, korormicin, thallusin or violacein (antifungal, antibiotic, morphogenesis and photosynthetic activities, respectively) in the defence response in macroalgal biofilm [53, 142–144]. In the communities associated with green, brown or red macroalgae, 12% to 50% of strains were able to produce antimicrobial effects in one or more target microorganisms [72, 74, 75, 140]. Remarkable was the discovery of the antidiatom activity produced by 80% of the strains isolated from *Ulva lactuca* against the diatom *Cylindrotheca fusiformis* [145] and 72% of the strains isolated from *Ulva reticula* against the diatom *Nitzschia paleacea* [24].

The most recent publications concerning the biotechnological potential and bioactivity production of microorganisms living in macroalgal biofilms will be referred below.

Two species of *Streptomyces* isolated from the brown macroalgae *Fucus spiralis* and *Cystoseira baccata* allowed the isolation of the following bioactive compounds: daunomycin, cosmomycin B, galtamycin B (antitumor and antibiotic activity); maltophilins (antifungal), and lobophorins (anti-inflammatory and antituberculosis) [146]. Compounds capacity of more than one activity has already been described [141]. Some alkaloids can even show quadra-activity. The study of marine bacteria and fungi from different sources led to the isolation of one bacterial strain (BMA6) from macroalgae with low activity against *Vibrio* sp. P3b [147]. The isolation of 31 Gram-positive and pigmented bacteria from Antarctic macroalgae allowed the selection of five strains phylogenetically related to *Agrococcus*, *Brachybacterium*, *Citricoccus* and *Kocuria*, that showed inhibitory effects, although without broad antibacterial spectrum, in the antagonism assay performed against other resident bacteria [148]. The cytotoxic and antibiotic compound cytochalasin D was, for the first time, isolated from a marine source, an endophytic *Xylaria* sp from the red algae *Bostrychia tenella* [149]. Susilowati et al. [150] isolated from *Sargassum* a bacterial strain with 95% similarity to *Bacillus subtilis* with high levels of inhibition against *Staphylococcus aureus* MRSA and *Staphylococcus epidermidis*. A study on green, red and brown macroalgae endo- and epiphytes revealed that 25% of the isolated epiphytes were able to produce inhibition against *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and *Candida albicans* (ATCC 90028) [151]. In this study, the red algae were the ones providing more bioactive strains.

Striking is the lack of reports of epiphytic fungi regarding bioactive production, in disparity to bacteria and endophytic fungi. Godinho et al. [128] isolated 148 fungi from the Antarctic macroalgae, *Monostroma hariotii* and *Pyropia endiviifolia*, of which two *Penicillium* strains were able to produce antifungal, antiprotozoal and cytotoxic compounds. Furthermore, 239 fungi were isolated from the same macroalgae and 6 showed between 61 and 96% bioactivity against selected targets, with even better results than the positive control against yellow fever virus [152]. A pseudodeflectusin compound produced by an *Aspergillus pseudodeflectus* associated to *Sargassum fusiform* showed to induce cytotoxicity in stomach and cervix human cancer cell lines [153].

Additional to the pharmacological applications of compounds produced by members of the macroalgal biofilm, another potential application is the usage of anti-settlement compounds produced by some strains in paints used in aquatic environments that will inhibit the adhesion and settlement of algae in the surface of boats and other objects.

Although in this genomic era there is an incredible increase of information about microbial communities, it is foreseen that only 1–5% of the microorganisms are able to be cultivated. However, several advances in the search for genes encoding secondary metabolites biosynthetic pathways by culture-independent methods, like metagenomics analysis and metabolomics, and application of this information in synthetic microbiology is increasing the possibilities to reveal new drugs impossible to discover until now. Unexpectedly, as opposed to what is found in sponge's bioactive studies, few are the genomic searches for genes that encode for polyketide synthetases or nonribosomal peptide synthetases in macroalgae associated microorganisms.

The discovery of new compounds and new bioactive producers open us possibilities to fight against emergent and still incurable diseases and provide new clues to the understanding of the ecological role played by the complex macroalgal biofilm communities that live under constant societal and environmental pressures.

5. Conclusion

Macroalgae are protected microniches prone to epibiosis by microorganisms where complex and highly dynamic interactions occur. Firstly colonized by bacteria, many other microorganisms which include microalgae like diatoms, fungi and protozoa constitute these biofilms.

Awareness of the importance of macroalgae and their biofilm has risen recently and, in the last years, we are gaining knowledge on its diversity, especially on the bacteriome, on the multiple functions played by both components of the holobiont, on macroalgal diseases and on the biotechnological potential of these communities. As only a low number of bacteria have been cultivated, we still have a relevant ecological potential to discover in many unknown bacteria. Furthermore, only very recently the world of macroalgal associated viruses started to be revealed. New methodological advances, metagenomics associated with metabolomic/proteomic studies will certainly foster our comprehension of the community structure and functioning of the microbial–macroalgal system. As only a very small part of the more than 35,600 different known species of macroalgae have been studied, we still have a long way to discover the hidden microbial diversity in their biofilms and its biotechnological potential, to understand all potential interactions between algal host and its microbial community, and the regulatory mechanisms in the extra coat of macroalgae.

Acknowledgements

We are grateful to Manuela Portugal d'Oliveira and Aires Oliva Teles for the critical review of this chapter. This research was partially supported by the Structured Program of R&D&I INNOVMAR – Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, Research Line NOVELMAR), funded

by the Northern Regional Operational Programme (NORTE2020) through the European Regional Development Fund (ERDF).

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