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REVIEWS

Review

The Macroalgal Holobiont in a Changing Sea

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When studying the effects of climate change on eukaryotic organisms we often oversee a major ecological process: the interaction with microbes. Eukaryotic hosts and microbes form functional units, termed holobionts, where microbes play crucial roles in host functioning. Environmental stress may disturb these complex mutualistic relations. Macroalgae form the foundation of coastal ecosystems worldwide and provide important ecosystem services – services they could likely not provide without their microbial associates. Still, today we do not know how environmental stress will affect the macroalgal holobiont in an increasingly changing ocean. In this review, we provide a conceptual framework that contributes to understanding the different levels at which the holobiont and environment interact, and we suggest a manipulative experimental approach as a guideline for future research.

Highlights

Macroalgae and their associated microbiota form a functional unit termed 'holobiont', characterized by its complex mutualistic relations. This symbiosis between macroalgae and the microbiota may be disturbed by environmental stressors, resulting in holobiont break-up.

Current knowledge on the functional consequences of the macroalgal holobiont is limited. Although it becomes increasingly clear that the microbiota is essential for host functioning, bacteria are still considered contaminants rather than a crucial component of the holobiont, and current studies are predominantly focused on the host only.

Macroalgae fulfil an important ecological function, and the effect of climate change on macroalgae has been a primary focus in recent algal research; however, the role of the microbiota in the hosts' response to climate change has not yet been addressed.

The Holobiont: A Functional Unity

Seawater contains vast numbers of microorganisms, with up to 10^7 viruses, 10^6 bacteria, and 10^3 microalgae per ml [1]. Many of these microbes form biofilms on a range of eukaryotic organisms, such as corals, sponges, and macroalgae. The microbial communities associated with different hosts contain a diverse assembly of organisms (including: archaea, bacteria, fungi, microalgae, protozoa, and viruses; Box 1), but differ markedly from the assemblages in seawater [2]. In fact, together, the host and the associated microbes are increasingly regarded by some authors as a functional unity called a **holobiont** (see Glossary) (for recent reviews on the holobiont concept and evolutionary perspective, see Bordenstein and Theis [3], McFall-Ngai et al. [4], Rosenberg and Zilber-Rosenberg [5], and Agler *et al*. [6]). This single ecological unit is a complex entity with highly specialized symbiotic interactions that are regarded as important in the functioning of all the organisms involved [7,8].

Macroalgae play a vital role in ecosystems as foundation species and ecosystem engineers. They provide food, shelter, and habitat for higher trophic levels and are responsible for a major part in the total primary productivity of temperate, arctic, and tropical systems around the world [9]. In addition, they have high economic value due to their application in aquaculture and biotechnology [10]. Today, we know that microbes often play a crucial part in macroalgal health, functioning, and development during the host's various life cycle stages through **mutualistic** interactions [11,12]. The interplay of macroalgae with their microbial component affects – among other things – nutrient exchange, defence mechanisms, morphology, reproduction, and settlement [13–15] (Box 2). Without their associated **microbiota**, macroalgae likely cannot function optimally, indicating that the association influences the provision of ecosystem services. However, microbes can also induce diseases, especially under changing environmental conditions, such as increasing seawater temperatures and ocean acidification [16,17]. Here, environmental stressors alter the microbial communities and disturb the symbiotic relation, which results in **holobiont break-up** [18,19]. With the cascading effects of a holobiont break-up and **dysbiosis**, the results of these changing conditions may be more severe than predicted. However, we also know that interactions between species may shift from negative to positive during increasing amounts of stress [20]. Thus, an increasing positive importance of mutualisms, facilitative interactions, and cooperative behaviour

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Box 1. The Underrepresented Components of the Macroalgal Microbiota

The majority of microbial-algal studies focus on bacteria. However, while metagenomic and transcriptomic research has indicated that microbial division is dominated by bacteria [28], other members of the microbial community may also play a significant role in the functioning of the holobiont [93]. Very little is known about the relationship between the macroalgal host and archaea, microalgae, protozoa, fungi, and viruses as colonizers. Viruses, in particular, have a large potential effect, as they could not only infect the host, but also other microbes, such as bacteriophages that regulate bacterial community composition, thus adding an extra complex interaction to the holobiont [94]. Even though viruses are extremely abundant in seawater, and contain high genetic diversity, the majority of previous research focused on merely a few small, filamentous brown algae, predominantly *Ectocarpus* spp. and *Feldmannia* spp., which are known to contain double-stranded DNA viruses [95–97]. McKeown *et al*. [98] discovered these viruses were present in kelps belonging to the genera *Laminaria* and *Saccharina* as well, and reported that two-thirds of the host populations were infected, highlighting the importance of incorporating viruses in studies on the holobiont. Recently, Beattie *et al.* [99] also detected novel single-stranded DNA viruses in the kelp *Ecklonia radiata*. The first characterization of a full viral community and the isolation of RNA viruses was completed by Lachnit *et al*. [100] on a red alga, *Delisea pulchra*. The virome from this study contained both epiphytic and endophytic viruses and included viruses that infect diatoms and fungi, and viruses that induce cell lysis. Viruses may spread diseases among host species or may be part of the holobiont immune system and can thus have both positive and negative impacts on the holobiont.

Another underrepresented topic in the macroalgal holobiont is the presence of **endophytic communities**. Most of the effort has concentrated on siphonous green algae (unicellular, multinuclear macroalgae), in which the endophytic communities were shown to be distinctly different from both the epiphytic communities and the surrounding planktonic communities [62,101–103]. Endophytic associations increase the complexity of the holobiont, because before a bacterium can enter the macroalgal thallus, it needs to damage the algal cell wall, which simultaneously provides entrance to pathogenic and opportunistic bacteria. On the other hand, endophytic bacteria can benefit the host by supplying nutrients and by detoxification. *Caulerpa* spp., for example, contain endophytes within the stolons (root-like structures) that take up phosphorus and nitrogen, explaining why these algae flourish in oligotrophic systems [104]. Similar mechanisms were found in *Codium decorticatum* and *Codium fragile* [101,105]. More so than for epiphytic communities, however, the ecological function of endophytes remains unexplored.

may also provide holobionts with additional resilience to changing conditions (stress gradient hypothesis; *sensu* Maestre *et al*. [21]). In the context of environmental changes, especially those that are human-induced, the holobiont concept becomes increasingly important.

Box 2. The Functioning Holobiont: Types of Interaction

The interactions between macroalgae and the microbiota are extremely diverse. Ranging from mutualistic to commensal and parasitic, the microbiota can be fundamental or detrimental to the functioning of the host [12]. The algal surface is a habitat that is ideal for the growth of the microbiota, as it is rich in organic carbon, oxygen, and nutrients [13–15,93]. In turn, the microbiota, for example heterotrophic bacteria and nitrogen–fixing cyanobacteria, may provide the algal host with CO_2 and nitrogen [12,39]. However, the functioning of the holobiont extends far beyond exchanging key nutrients.

Microbial communities form a biofilm on the host, which acts as both a physical and a physiological barrier between the host and the environment [11,50]. This barrier can modulate the availability of resources for the host, having an 'insulating' effect. The insulating effect can also be positive, as the biofilm can protect the host against toxins, heavy metals, and UV radiation, by adsorption or even transforming the toxins to less harmful compounds [11,14,106]. Microbes are an important factor in the early phases of macroalgal life history as well. The amount of bacteria in the biofilm on surfaces (e.g., rocks) has a positive effect on the number of zoospores settling on the substrate [107], as does the age of the biofilm [108], thus facilitating the successful colonization of new surfaces by macroalgae and the formation of new holobionts [14]. In addition, the release of spores can be influenced by biofilms due to the secretion of secondary metabolites that act as a cue [73,109]. Finally, the biofilm has an important chemical function. Certain algae-associated bacteria may play a part in the host defence strategies against unwanted microbes and biofouling by outcompeting other bacteria. They can, for instance, secrete antifouling chemicals and antibiotics, thus maintaining the health of the host [14].

However, harmful microbes, including viruses, eukaryotic parasites, and bacteria, are also associated with macroalgae. Pathogenic bacteria and fungi may cause rot symptoms and diseases such as bleaching. However, due to the fact that pathogenicity is often associated with the degradation of the cell wall, it is hard to distinguish true pathogens from saprophytic epiphytes [110]. As many bacteria and fungi exhibit the potential to degrade algal cell walls, it should be noted that bacteria at first might be commensal and only later become harmful as a result of changes in the environment [12].

Glossary

Axenic host: a single species that is free from an associated microbiome/microbiota or other epiphytic and endophytic organisms.

Climate change: change in the state of the climate over an extended period (mainly the alteration of the composition of the atmosphere), which can be part of the natural climate variability (e.g., due to solar cycles) or caused by persistent anthropogenic pressure. In this review, climate change is used in the latter sense.

Dysbiosis: a shift in the microbial community with negative consequences for the host.

Endophytic communities: microbial communities living within the macroalgal thallus.

Epiphytic communities: microbial communities associated with the macroalgal surface.

Functional guild: a group of organisms that is functionally similar, for example based on the presence of functional genes. Members of the same guild are not necessarily taxonomically related.

Holobiont: an assemblage of different organisms forming an ecological unit (often defined as the host organisms plus all of its microbial symbionts).

Holobiont break-up: the disturbance and disruption of the mutualistic association between host and microbiota. This can be caused by environmental stress.

Microbiome: the collective genomes (genetic material) in a community of microorganisms (e.g., bacteria, archaea, fungi, viruses, protozoa).

Microbiota: an ecological community of microorganisms (e.g., bacteria, archaea, fungi, viruses, protozoa).

Mutualism: an interaction that is beneficial to all parties involved. The mutualistic interaction can be obligate (the species are dependent on each other) or facultative (the species are not fully dependent on each other and can survive without the benefits of mutualism).

Oxidative burst: the rapid release of harmful reactive oxygen species (e.g., superoxide ions and hydrogen peroxides).

The anthropogenic pressure on coastal ecosystems is large [22]. Elevated atmospheric CO_2 concentrations (i.e., the alteration of the composition of the atmosphere – **climate change**) cause seawater temperatures and seawater CO_2 concentrations to rise. By the year 2100, seawater temperatures are predicted to increase 2°C, CO_2 concentrations to increase 200%, and the pH to drop 0.2 units, under business-as-usual modelling projections (scenarios in which no additional efforts to counteract climate change are assumed; that is, Intergovernmental Panel on Climate Representative Concentration Pathway scenarios 6.0 and 8.5) [23]. In addition to climate change, human activities decrease the amount of suitable substrates available (habitat destruction), pollute sediments and waters, and alter other environment factors such as the amount of solar UV radiation and nutrient concentrations [22].

In the context of climate change, it has been shown that macroalgae – a diverse paraphyletic group – will show a wide variety of responses to elevated temperature and ocean acidification [24,25] (Box 3). Although both the commercial application and ecological services provided by macroalgae are, in many cases, highly dependent on the interactions with their microbiota (due either to the benefits received by the microbial community, or to detrimental effects caused by the microbes, such as disease), there is only a rudimentary understanding of the functional consequences of the holobiont and how it responds to climate change [26]. Thus, integrating the holobiont concept in future research is essential. The aim of this review is (i) to identify knowledge gaps in our

Box 3. Macroalgal Responses to Climate Change

Macroalgae are a diverse paraphyletic group with many different functional forms, ranging from calcified algae to turf-forming mats and leathery kelps [111]. Consequently, they exhibit an array of responses to changing environmental conditions. Climate change mainly impacts macroalgae via elevated seawater temperatures and increased CO_2 concentrations. Whereas the former environmental factor is relatively easy to measure, the latter causes complex changes to the seawater carbonate system, ultimately resulting in a decrease in pH (i.e., ocean acidification) [23]. Coralline algae form a group of species highly vulnerable to ocean acidification (calcification rates are reduced due to a decrease in carbonate ions) [24]. On the contrary, as CO_2 is the required substrate for carbon fixation, several fleshy macroalgal species can benefit from ocean acidification (e.g., increased growth or elevated photosynthetic rates) [25]. Other fleshy species show no response to elevated CO_2 [25], or experience detrimental effects as they are sensitive to increases in $[H^+]$ [112]. Elevated temperatures impact metabolic activity as well as growth rates and reproduction. Thermal stress causes slower growth rates [113] and decaying tissue [114] at sublethal temperatures. However, ocean acidification is able to reverse the negative impact of elevated temperature in some cases, showing the importance of synergistic effects [56]. Apart from growth and photosynthesis, ocean acidification and temperature also affect biochemical composition (e.g., lipids, proteins, pigments) in various ways [115].

On a larger scale, these varying responses have biogeographical and ecological consequences. Temperature, for example, is one of the most important factors in determining biogeographic distribution, which may result in range expansion for some species, and the local extinction of other species [116]. Due to the negative effects of ocean acidification and temperature, calcifying species and kelps may decrease in abundance, biomass, and diversity, whereas turf-forming species are likely to increase, leading to a loss of habitat-forming species and a reduction in the complexity of the system [117,118]. This shows that the effects of environmental change are not only about the single effects of pH and temperature, but perhaps more importantly concern changing interactions between species across trophic levels. Increasing temperatures, for example, not only influence the biogeographic range of kelp, but also the distribution of fish and urchin grazers, which has resulted in the dramatic reduction of kelp forests in the past few years [119]. Other human impacts (e.g., eutrophication, increased UV radiation, overfishing) may also result in altering of the community structure and regime shifts, for example from reef-building calcifiers to coral-overgrowing filamentous algae [120]. Whereas bacteria–macroalgae interactions can be easily studied in the controlled conditions of the laboratory, interactions between macroalgae and higher trophic levels can typically not be confined to small experimental set-ups. This difference in scale likely requires an approach different from the $CO_2 \times$ temperature cross experiments.

Closely linked to the impact of climate change on physiology is the concept of macroalgal health. Relatively little is known about the prevalence and magnitude of diseases in macroalgae [94], but they are predicted to increase under elevated environmental stress [16], or could change distribution and virulence. Diseases directly impact the infected organism and population, but may additionally have cascading effects on the ecosystem if keystone species are infected [19,94]. This again underlines the importance of a holobiont approach.

understanding of the interaction between the environment and the macroalgal holobiont; (ii) to provide a framework to interpret the interactions between environment, host, and microbiota; and (iii) to provide a scope for future studies. In the first part of this review we briefly describe those factors which impact the formation of microbial communities on macroalgae, highlighting the importance of the environment in shaping these interactions. In the second part, we examine what is known about the impact of climate change on the macroalgal holobiont. Finally, we describe a framework for interpreting the interactions between the macroalgal holobiont and the environment, and we highlight a way forward for future research.

On the Selection of Communities

Most of the previous research on the macroalgal holobiont focused on describing and characterizing the bacterial communities at a phylogenetic and functional level [27–29]. Comparable with seawater, microbial abundance on macroalgae is high, ranging from 10^6 to 10^7 bacteria per cm^2, with a high phylogenetic diversity [7,12]. The bacterial communities associated with the host differ markedly from the bacterial assemblages in seawater, as well as from biofilms that form on non-living substrata [2,30–34]. This indicates that macroalgae modify and influence the assembly of their own bacterial community to a certain extent, or that the bacterial community selects its host. In accordance with this finding, studies by Lachnit *et al*. [35], Nylund *et al*. [36] and Bondoso *et al*. [37] showed that different macroalgal species growing at the same location have different bacterial communities in terms of abundance and composition, whereas individuals from the same species growing in different habitats have similar bacterial communities. In other studies, however, there was a high variability in bacterial species composition among individuals from the same species [30] or the bacterial communities were mainly influenced by site-specific environmental conditions [38]. Thus, while some macroalgae seem to attract species-specific communities of microbes through holobiont-specific interactions, other mechanisms play a significant role in the formation of macroalgal–biofilm communities.

Phylogeny versus Function

Certain bacterial taxa, mostly at the phylum level, are commonly found in algal communities (e.g., Gammaproteobacteria, Alphaproteobacteria, and Actinobacteria) [39,40]; however, ecologically relevant similarities on a lower taxonomic level are virtually absent [12,39]. Rather than selecting bacteria that are closely related, the mechanisms underlying colonization of macroalgae seem to be driven by functional similarity, that is, through the selection of bacteria with similar functional traits. Evidence for this hypothesis was supplied in a study using metagenomics on the green macroalga *Ulva australis* Areschoug, which showed that the phylogenetic similarity of the bacterial community across the host species was low, while the functional composition was similar [41]. Microbes are very important for the morphological development (morphogenesis) of *Ulva* spp., and without specific bacterial signals *Ulva* spp. tend to develop into undifferentiated cells [42]. When these atypical colonies are subsequently provided with appropriate microbial communities – not necessarily with the same taxonomic background, but instead with similar functional characteristics – the algae will develop their typical morphology again [43]. Another metagenomics study by Aires *et al.* [44], on the red macroalgae *Asparagopsis taxiformis* (Delile) Trevisan and *Asparagopsis armata* Harvey, linked environmental characters to metabolic traits of the associated microbes, rather than taxonomy. These studies suggest that initial colonization is subject to chance (according to the lottery model), that is, the species that arrive first will be the first to colonize, on the condition that they possess the functional traits required to successfully colonize [45,46]. When this applies, the final microbial community does not necessarily need to be phylogenetically related, but instead will consist of functionally similar bacteria (belonging to the same '**functional guild**'). A study by Roth-Schulze *et al.* [47] showed that the majority of **epiphytic communities** belongs to functional guilds that contain genes required for successful epiphytic colonization (e.g., adhesion, chemotaxis) and life in a biofilm (e.g., the ability to use polysaccharides and quorum-sensing regulation). To a smaller extent, there are also host-specific assemblies that have adapted to certain host characteristics. *Caulerpa filiformis* (Suhr) Hering for example, a species with a high polysaccharide xylan content, has microbial communities that are enriched in enzymes related to xylan-degrading enzymes, compared with other macroalgal species that do not contain xylan in high amounts. Similarly, the microbial communities of the brown macroalgae *Ecklonia radiata* (C.Agardh) J.Agard and *Phyllospora comosa* (Labillardière) C.Agardh were overrepresented by enzymes involved in the breakdown of the high content of *N*-acetylgalactosamine [47]. This also shows why macroalgae that are taxonomically closely related do not necessarily have microbiota and **microbiomes** that are similar (either functionally or taxonomically), as the host-specific assemblies may be selected based on the characteristics of the macroalgal species and the environment [33,40,47,48]. The traits needed by the microbiota to successfully colonize a host may fluctuate, however, as environmental and chemical conditions change, such as the pH, oxygen concentration, and presence of secondary metabolites. The algal host could be considered a niche, and certain algal species or environmental conditions could be able to modify the bacterial communities, thus exerting selective pressures [41]. As function is more important than phylogeny, rather than focusing on the taxonomic diversity of microbial communities, future research should aim to increase our knowledge on the diversity of the microbiome's functional genes.

The Host and the Environment

Macroalgae contain carbon- and nutrient-rich polymers (e.g., agar, carrageenan, and cellulose) and produce oxygen as a by-product of photosynthesis, therefore representing a suitable niche for many microbes such as aerobic and polymer-degrading bacteria. For example, a study by Barott *et al*. [7] showed that the majority of bacteria associated with algae were obligate aerobes and photoautotrophs, whereas the bacteria colonizing corals were mostly facultative anaerobes and heterotrophs. Contrary to favouring specific functional guilds of bacteria, macroalgae can also repel microbes by using '**oxidative bursts**' and secondary metabolites as a defence mechanism. Thus, on a micro-scale, macroalgae modify their immediate environment in response to physiological processes and defence mechanisms. On a larger scale, epiphytic communities may also be influenced by external environmental conditions, such as light availability, nutrient concentrations, and pollution (e.g., large amounts of sewage water or heavy metals) [38,49,50]. Brodie *et al*. [27], for example, found a higher diversity of microbes in the upper regions of the intertidal area than on the middle and lower shore. This is possibly due to the higher environmental stress on the upper shore, for competition may reduce the diversity at lower levels of disturbance. In addition, the pelagic community – and therefore the pool from which macroalgae may be colonized – is influenced by environmental conditions [51,52]. It is clear that many factors, and both the host and environment, are important in the colonization process and assembly of microbial communities.

Changing Communities in a Changing Environment

Several studies have tested for the effect of the environment on macroalgae holobiont by comparing the microbial community composition in different habitats [44,49,53], but few studies have so far incorporated manipulative experiments. A major conclusion from the available experimental manipulations is the potential of the biofilm to modify host responses to changing environmental conditions [54–56].

Saha *et al*. [54] in a mesocosm experiment investigated whether the brown fucoid alga *Fucus vesiculosus* Linnaeus responds to light limitation and temperature shifts by changing the production of its antifouling compounds and whether that affects the epiphytic microbial communities. Incubation at 25°C induced severe stress of the thallus (i.e., decaying apical tips), compared with temperatures between 5°C and 20°C. Shading significantly reduced carbon fixation with a linear relationship. Whereas the effect of environmental stressors on antifouling compound concentrations was limited, the stress led to changes in bacterial community composition and richness but not in abundance.

Another mesocosm experiment by Mensch *et al*. [55] exposed *Fucus vesiculosus* forma *mytili* to elevated pCO_2 (carbon dioxide partial pressure) levels and increased temperatures. The bacterial community composition was strongly affected by temperature and significantly – albeit weakly – influenced by elevated pCO_2. Especially interesting was the higher relative abundance of potential pathogens under increased temperatures, which was correlated to the reduced biomass of the host, indicating that pathogens may play an important role in combination with thermal stress. On the contrary, elevated pCO_2 levels enhanced the growth rates of *F. vesiculosus* f. *mytili*, even in combination with increased temperatures. With increased CO_2 concentrations, a shift towards bacteria that are naturally more adapted to high pCO_2 levels was observed (these bacteria are commonly found in deep-sea sediments and on tidal flats). Similarly, a mesocosm study on the giant kelp *Macrocystis pyrifera* (Linnaeus) C.Agardh by Minich *et al.* [56], showed that kelp growth was negatively correlated by elevated temperature, whereas a combination of elevated temperature and elevated CO_2 concentrations resulted in positive growth. Evidently, for both *F. vesiculosus* and *M. pyrifera*, synergistic effects (temperature versus pathogens, and temperature versus pCO_2) appear to be important, which has also been noted during physiological experiments solely targeting macroalgae [57,58]. The lower growth rate in *M. pyrifera* under increased temperatures was associated with a shift in the microbial composition: the Alteromonadales declined in abundance, whereas the Flavobacteriales increased. Bacterial strains of the latter order are often connected with diseases in red and brown macroalgae, as well as with bacteria containing alginate lyase enzymes, which catalyse the degradation of alginate present in the kelp tissue [56].

Shifts in microbial community in response to high temperatures have also been observed on crustose coralline algae (reef-building algae that fulfil an essential role in coral health). Webster *et al*. [59] showed that, when seawater temperatures increased to 32°C, these calcified algae experienced bleaching (loss of pigmentation) and reduced maximum quantum yields (an indicator of the performance of photosystem II), together with a shift in the microbial community structure. Temperatures lower than 32°C also led to bleaching and reduced maximum quantum yields but no shifts in microbial community structure. Interestingly, algae cultured at <32°C recovered when temperatures were reduced to the ambient level (27°C), but this was not the case for algae cultured at 32°C. Controversially, a study on the effect of near-future ocean acidification conditions on rhodoliths showed that the associated microbiota remained stable (whereas the communities in both the seawater and on dead rhodoliths shifted) [60]. The algae showed no sign of calcium carbonate biomass loss, and even increased photosynthetic activity. This resilience to environmental stress was likely provided by stability of the microbiome [60].

Another relatively stable microbiota, despite the combined impact of ocean acidification and ocean warming, was observed in the green macroalga *Caulerpa taxifolia* (M.Vahl) C.Agardh [61], together with in increased growth rates of the host in response to these changing environmental conditions. Only one out of 1087 bacterial operational taxonomic units (OTUs) increased significantly in abundance when both CO_2 concentrations and temperature were elevated. This OTU belonged to the genus *Planctomyces*, which is known to be very adaptable to different environmental conditions, and the authors state that this OTU could play a role in the response of *C. taxifolia* to climate change. However, only epiphytic communities were measured in this study, and as *Caulerpa* species are siphonous, they are known to have extensive endophytic communities with tight associations [62,63] (Box 1). To be able to fully apprehend the effect of a changing environment, and the resilience that the microbiota may provide, these endophytic communities should be included.

Interpreting the Interaction between Environment and Holobiont

From the large amounts of climate-change-related research on macroalgae, only a handful of studies have incorporated the microbiota and even fewer the holobiont concept. These pioneering studies in the examples above indicate that environmental conditions reflecting climate change affect the entire holobiont (both the microbial communities and the host), and revealed that bacterial communities may be able to remain stable or adapt, contributing to the resilience or resistance of the holobiont, respectively, but that the effects for the host can also be negative. Nevertheless, these results do not infer causality or whether the shifts in microbiota are a consequence of experimental shifts or the cause of the observed response in host physiology. So far, the mechanisms driving holobiont interactions remain largely unknown. The consequence of this fundamental lack of knowledge is that we cannot evaluate the significance of the host–microbiota interaction in the context of the ability of macroalgae to withstand (resistance, resilience) change or to adapt to new environmental conditions. Moreover, distinguishing between cause and effect is difficult as all three components – environment, host, and microbes – may interact with each other simultaneously. Thus, before the role of the holobiont in relation to climate change can be assessed, a conceptual framework is needed to interpret the level at which interactions take place.

A Framework

We propose that climate change interacts with the holobiont on three basic levels (summarized in Figure 1, Key Figure): (i) direct effect on microbiota, (ii) direct effect on host physiology, and (iii) mediated effect on host–microbiota interactions.

(i) Changing environmental conditions may have a direct effect on the microbial community traits of the host-associated microbiota. First, the shifting of environmental conditions expected under climate-change scenarios might lead to fluctuations in the microbial composition in the seawater column [51,52]. This will, in turn, affect the microbial community that can colonize the host and the functional traits associated with the community (e.g., microbial communities might shift in terms of absolute abundance, relative abundances, and species richness, ultimately resulting in different functional guilds).

(ii) Climate change may directly affect the physiology of the host, for example, the enzymatic activity, photosynthetic rates, growth and reproduction, with ulterior changes in the functioning, performance, and survival of the host (Box 3). We want to emphasize that not all components of environmental change are necessarily stressors. The increased level of CO_2, for example, also increases the amount of substrate available for photosynthesis, resulting in increased growth in certain fleshy macroalgae [25].

(iii) Climate change may also have a mediated effect on the interactions between host and microbes, as microbial communities with a different functional guild affect the host differently, and vice versa a host with a changed physiology provides a different kind of niche to microbes. For example, an environmentally induced shift towards an epiphytic community with a higher abundance of pathogenic microbes [55,56] may subsequently be detrimental to the host. In turn, reduced photosynthetic activity due to a decreased pH, and thus a lower production of oxygen by the algal host [64,65], could potentially lead to the algal surface being an unfavourable habitat for aerobic bacteria. These are just two of many hypothetical examples of how climate change can have a mediating effect on host–microbe interactions, and thus, illustrates the complexity of studying the response of the holobiont to changing environments.

Key Figure

Overview of the Interactions between the Host, the Microbial Community, and Climate Change

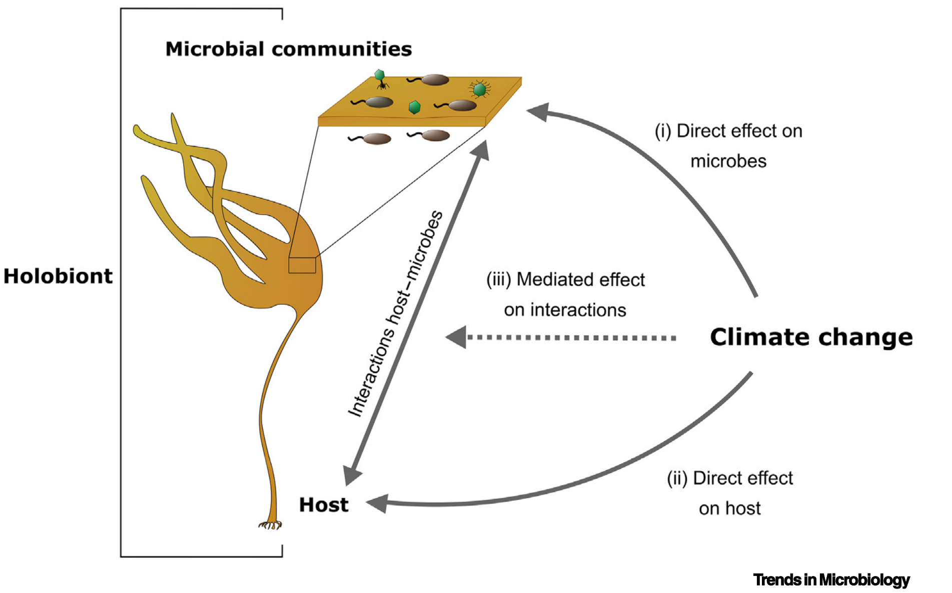

Figure 1. Climate change (e.g., stressors or changes in environmental conditions) will separately have an effect on (i) the microbes associated with the host, and (ii) the host itself, causing changes in host physiology and microbial community traits. This will affect the interactions between the host and the microbial communities (iii), and thus the outcome of the symbiotic relationship.

The effect of climate change working on these three different levels (host, microbes, and interactions) will determine the functional outcome of the holobiont, that is, whether these interactions are beneficiary or detrimental (Box 2). From the framework presented here, we can draw different possible scenarios (summarized in Figure 2).

In the first scenario, host and microbiota are each directly affected by climate change [the interaction plays no role, i.e., climate change is acting mainly on components (i) and (ii) in the framework]. Here, the impact of climate change on the functional outcome of the holobiont can be positive, negative, or neutral. However, whereas host and microbiota may both react in the same way (e.g., both are positively impacted by environmental change; Figure 2A), they could also react differently (a mismatch). The overall outcome will then likely depend on the strongest driver, which may result in cascading reactions. If elevated temperatures, for example, negatively affect the host, but do not affect the microbiota, a negative cascade of reactions may result in an overall negative outcome for the holobiont (Figure 2B).

In the second scenario, the host–microbiota interaction plays an important role in the functional outcome of the holobiont [i.e., component (iii) in the framework], for climate change could either intensify the mutualism (positive effect on the holobiont; Figure 2C), or could degrade the mutualism (negative effect on the holobiont; Figure 2D) and ultimately result in holobiont break-up.

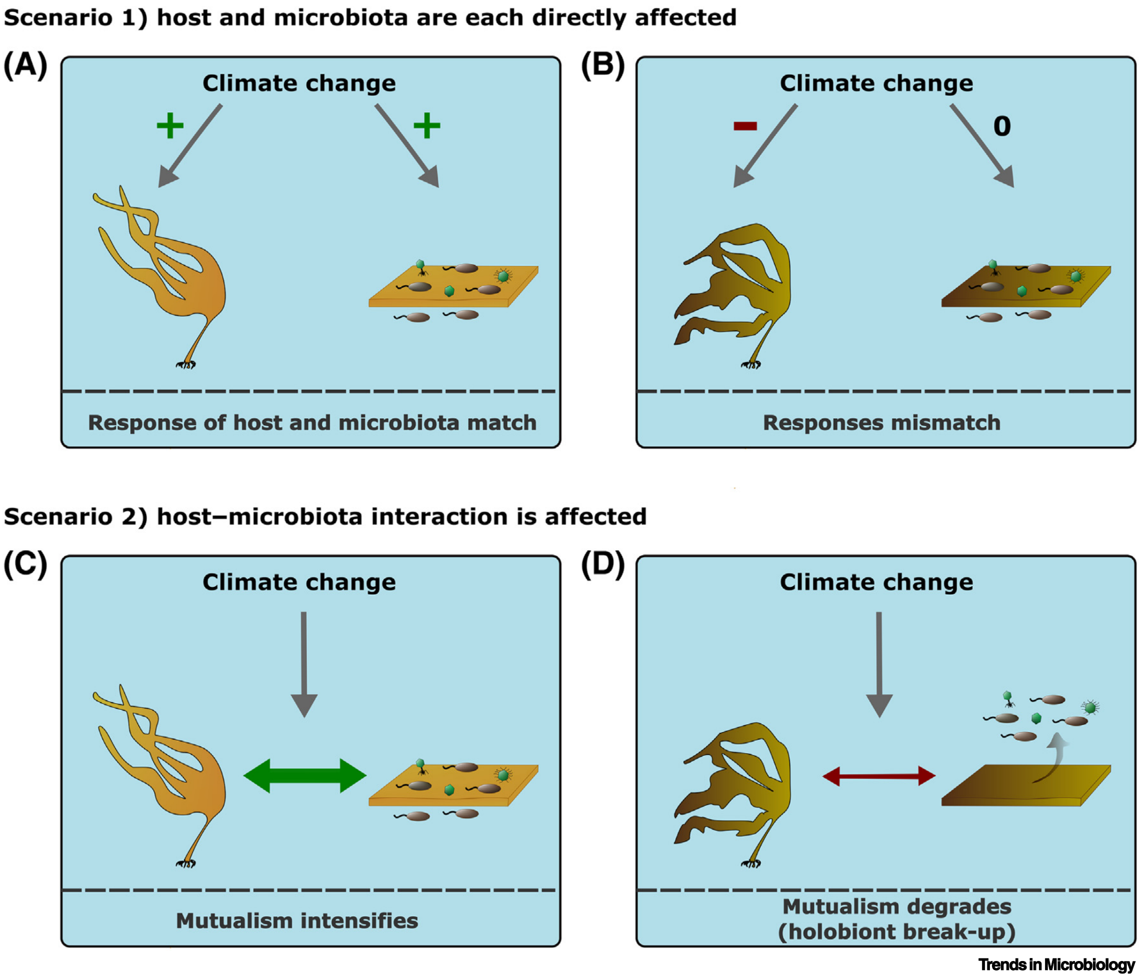

Figure 2. Overview of Possible Scenarios Describing How Environmental Factors Reflecting Climate Change (e.g., Elevated CO_2 Concentrations and Temperature) May Affect the Functional Outcome of the Holobiont. In scenario 1 (A+B), both host and microbiota are directly affected by climate change (which could be a positive, negative, or neutral effect). Here, the host and microbiota may respond similarly, for example, host and microbiota both react positively (A), or the responses could mismatch, for example, the host is negatively affected and the microbiota is not affected (B). In the latter, the functional outcome will likely depend on the strongest driver and may result in cascading reactions. Note that other combinations are also possible (e.g., both are negatively affected, or only the microbiota is positively affected), but are not shown here. In scenario 2 (C+D), climate change affects the host–microbiota interaction, which then shapes the functional outcome of the holobiont. Here, climate change may either intensify the mutualism (C), or it could degrade the mutualism (D), which may ultimately result in holobiont break-up. While most studies aimed to assess the effect of climate change on host and microbial communities separately (scenario 1), it is likely that host–microbial interactions played a role nevertheless, and those results are thus likely mixed with scenario 2.

While studies that characterize bacterial communities on both phylogenetic and functional level are important, because these provide insight on the diversity and functioning of the holobiont, they do not provide information on what level the interactions take place. Similarly, mesocosm experiments show that the holobiont changes in relation to changing environmental factors, but not how the environment influences the different levels of the holobiont individually. The effect of climate change on the (iii) component in the framework (host–microbial interactions) has received the least attention in current research, and yet will likely be the most important factor in determining the resilience of the holobiont in the face of climate change. Moreover, when studies report the effect of climate change on microbial communities [component (i)], or host physiology [component (ii)], it is often forgotten that host–microbial interactions take place. Thus, while most research aimed at determining the effect of climate change on the host and microbiota separately (Figure 2A,B), those results are likely influenced by host–microbial interactions and mixed with scenario 2 (Figure 2C,D). To be able to clearly establish causality, we need to extend beyond descriptive studies and instead incorporate more manipulative experiments, in which both the holobiont and environmental conditions are manipulated.

An Experimental Approach to the Impact of Climate Change on the Macroalgal Holobiont

There are many experimental approaches that can allow us to shed light on the macroalgal holobiont in relation to environmental change. These include laboratory and mesocosm experiments in which the environment is manipulated (e.g., increase or decrease in temperature, CO_2 concentrations, nutrient concentrations, or a combination of factors), as well as experiments in the field. From here, methods can focus on physiological changes in the host, phylogenetic or functional (i.e., metagenomic) changes in the microbiota and microbiome, or merging several approaches such as transcriptomic, proteomic, and metabolic responses of the entire holobiont [26,28]. Whereas all mentioned approaches are useful and can contribute to an understanding of the holobiont, manipulative experiments are currently predominantly limited to manipulating environmental factors, and very few studies take it as far as to manipulate the holobiont (but see e.g., Campbell *et al*. [66] and Case *et al.* [67] for exceptions).

Macroalgae collected from the field and directly used in experiments already contain a microbiota. Little is known on how fast microbial communities change, and presettled microbial communities may also excrete antibiotics or metabolic compounds, thereby influencing the functional outcome of the holobiont and the experimental results. Thus, these hosts cannot be considered 'clean slates'. A first step in a manipulative experiment in which not only the environmental parameters but the microbial component is manipulated as well, is therefore to culture macroalgae without an associated microbiota (i.e., an **axenic host**). This will allow us to truly differentiate between separate components of the holobiont and to understand the consequences of the microbiota for host functioning. While all different experimental approaches are viable and useful – including the use of nonaxenic hosts – we argue that implementing the use of axenic hosts on a larger scale in future studies will be a crucial step. Axenic cultures are import tools in studying and extracting various compounds released by macroalgae without contamination (including allelopathic compounds, genomic DNA, and cDNA libraries), in preparing seed-stock for aquaculture, and in studying the effect of bacterial morphogenetic compounds on macroalgae [68–70]. In addition, extensive axenic cultures can be derived from a single algal specimen, thus minimizing the variability between hosts when used in experiments. With regard to testing the effect of a changing environment, perhaps the most important asset of creating axenic hosts is that this enables the use of fully manipulated holobionts. By inoculating the axenic cultures with a particular set of microbes, or by supplying the hosts with seawater containing a selected microbial community (Figure 3), different experimental groups can be created. Those microbes can be chosen based on, for example, their function in morphogenesis [71], the potential to induce diseases [72], their function in immune system and production of compounds [73], or other functions of interest. Additionally, axenic cultures can be maintained under sterile conditions, and holobionts resembling *in situ* holobionts (realistic holobionts) can be created by supplying axenic hosts with nonsterilized natural seawater (Figure 3). Alternatively, macroalgal specimens from the field can be directly used instead of the latter experimental group. When using both axenic hosts and nonaxenic hosts in the same experiment, however, it is recommended to create holobionts using axenic hosts supplied with nonsterilized seawater, instead of directly using specimens collected in the field, as to exclude any effect of the pretreatment. Finally, comparing the reaction of the manipulated holobionts with axenic hosts to a changed environmental parameter (e.g., temperature or CO_2 concentration, described as 'climate change' in Figure 3) will give insight if and how the microbial community affects host performance.

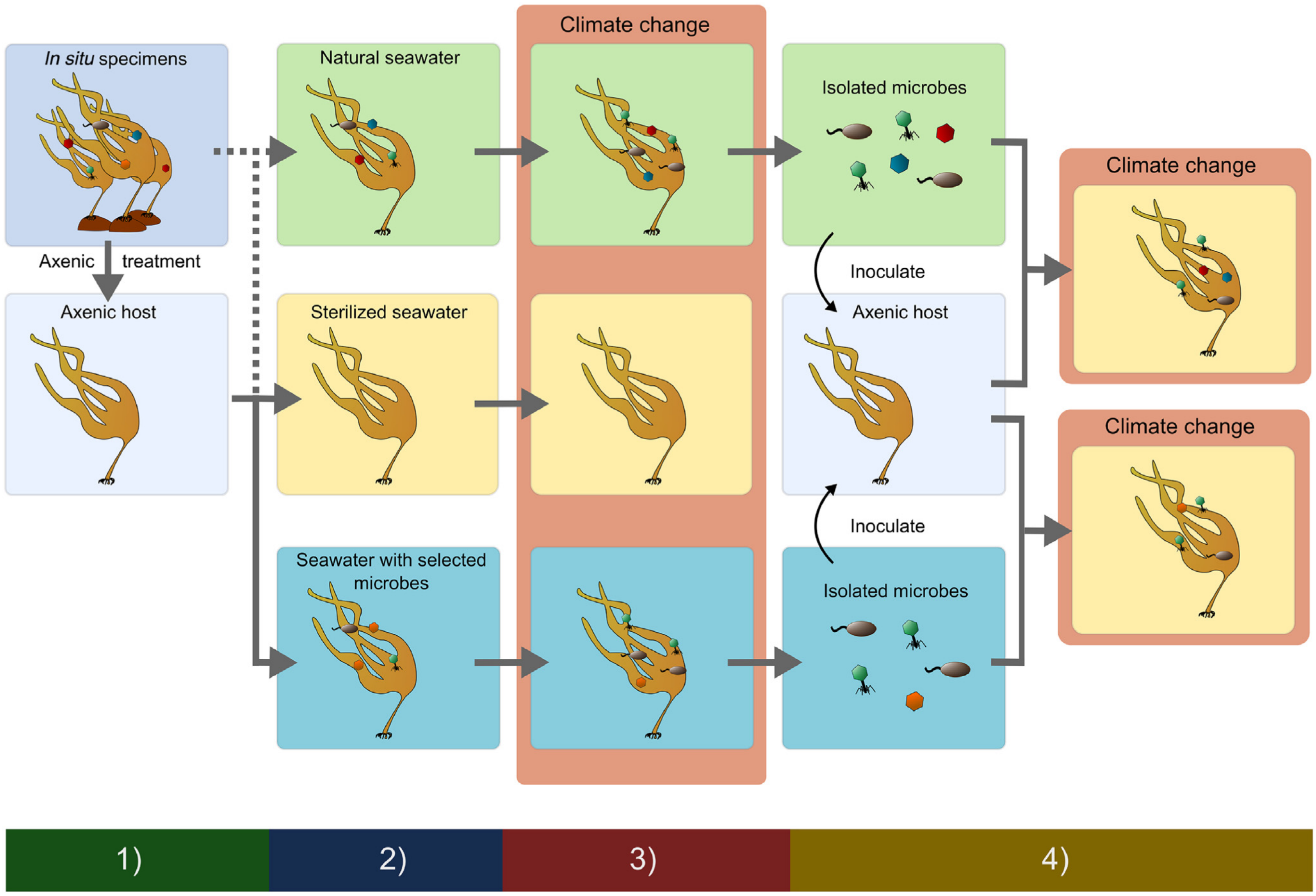

Figure 3. Schematic Outline of a Possible Manipulative Experimental Approach as a Guideline for Future Research. (1) Culture algae without an initial associated microbiota (i.e., an axenic host) by sterilizing specimens collected in the field or by isolating reproductive cells under sterile conditions. (2) Create different experimental holobionts by supplying one experimental group with sterilized seawater (maintaining axenic cultures) and two other experimental groups with seawater containing a selected community of microbes (creating a manipulated holobiont) or with nonsterilized natural seawater (i.e., creating a realistic holobiont resembling *in situ* specimens). Alternatively, specimens collected *in situ* can be directly used in the latter experimental group (alternatives are depicted in the figure with dashed lines). (3) Change an environmental parameter (e.g., elevated CO_2 concentrations or temperature) to analyse how macroalgae react to climate change with and without associated microbes. (4) Establish causal relationships by reinoculating an axenic host with specific communities of isolated microbes and checking whether a response similar to that in step (3) is triggered when environmental parameters are changed again.

Thus, such an experimental approach enables us to test the effect of the (iii) component of the framework defined in this review.

Axenic cultures can be established by either isolating the reproductive cells (e.g., gametes, tetraspores, zoospores) under sterile conditions, or by sterilizing the macroalgal tissue. Although the optimum method may differ between host species, often a mix between physical methods (e.g., sonication, brushing) and chemical methods (antibiotics, disinfectants) works best [74]. The different available treatments, as well as sterility tests, are summarized by Kawai *et al.* [68]. Whereas microalgal axenic cultures are quite common [75,76], macroalgal axenic hosts are currently not often used. This is likely due to the fact that obtaining truly axenic macroalgal hosts often proves to be difficult, and establishing such cultures takes time. Despite these difficulties, axenic hosts have reportedly been established for a variety of macroalgal species, for example, the red algae *Delisea pulchra* (Greville) Montagne [67], *Goniotrichum alsidii* (Zanardini) M.A.Howe [77] and *Polysiphonia urceolata* (Lightfoot ex Dillwyn) Greville [77], the brown algae *Sargassum* *polycystum* C.Agardh [78], *Fucus spiralis* Linnaeus [79], *Dictyota dichotoma* (Hudson) J.V. Lamouroux [80] and *Ectocarpus* spp. [69], and several kelp species belonging to the Laminariales [81–83], green algal species belonging to the genus *Ulva* [70], and even siphonous green algal species of the genus *Acetabularia* [84]. Solid proof of the acquired axenicity has been given in several of these studies (e.g., in the case of *Ulva* spp., *Ectocarpus* spp., and several Laminariales), using visualization methods such as staining bacteria with fluorescent dyes or by amplifying the 16S rDNA gene via PCR. The sterility of the protocols in several of the older studies (i.e., before the rise of molecular techniques) is, however, to be questioned, as the axenicity test is often missing [71]. When performing experiments using axenic cultures, true axenicity should be confirmed in all cases. Although obtaining axenic cultures certainly can be strenuous for some species, once an axenic culture is successfully established, it is often easy to maintain long term [68,69], and cryopreservation can allow for storage of the axenic hosts [85]. Developing protocols to create axenic stocks for a wider range of macroalgae can greatly increase the level on which we can perform manipulative experiments.

Removing an essential component of the holobiont may seem counterintuitive, and for some species this may be the case. In many *Ulva* spp. and *Ectocarpus* spp., for example, axenic hosts develop into an atypical morphotype [43,86]. However, upon adding the appropriate microbes, the algae recover their typical form. In this way, being able to create axenic hosts actually gives a better understanding of the function of the microbiota component. Alternatively, the natural epiphytic community can be accepted as part of the natural variation (as one would think of genetic variation in a population). This is ecologically more realistic (a macroalgal thallus in the field will never be axenic), but the disadvantage of such an approach is that the separate influence of components (i), (ii), and (iii) from the framework cannot be assessed individually. Thus, as generally is the case, the optimal methodological approach will depend on the research question.

Many hypotheses have been formed about the influence of the microbiota on the host's response to changing environmental variables (including the possible role they could play in resilience, as well as the possible increase in pathogens), but to date relatively little evidence has been given for these hypotheses. However, this may be due to the fact that few manipulative experiments have been carried out. To be able to conclude that a certain response to climate change in the host is triggered by the microbiome, a causative relation needs to be established. With regard to pathogen–disease relations, Koch's postulates are used, from which the third criterion states that reinoculation of the isolated microorganism into the host should lead to the expected phenotype if a causal relationship between microorganism and phenotype exists [87]. This criterion, however, is not only applicable to diseases and detrimental responses, but also to positive responses such as resilience. After assessing the response of the host in the different manipulated holobionts (discussed in the previous paragraphs), different functional guilds of interest can be selected. Upon inoculating new axenic hosts with these selected microbes only, and applying the same environmental conditions, a response similar to what was observed in previous experiments could be triggered in the host, thus fulfilling Koch's third postulate (Figure 3). If no response is observed, either other microbes are responsible for the response, or the interaction strength is affected regardless of microbiome composition. In addition, it is important to distinguish between the effect of cell-to-cell-based communication (i.e., the presence of the microbe on the algal surface is required) versus waterborne compound-mediated communication (i.e., response of the host is triggered by info-chemicals and does not require physical contact with the microbes itself). Differentiating between those can be done using, for example, a two-chamber system [71].

Another major challenge in unravelling macroalgal holobionts are field experiments. Whereas laboratory experiments are very suitable to manipulate and study the effects on host and holobiont separately, they do not fully mimic the complexity of natural ecosystems. Additionally, laboratory experiments only allow for short-term experiments, while the long-term effects will be important in studying the adaptive ability of the holobiont. As ocean acidification and elevated temperature are slow processes, assessing the effects on communities in the field is difficult. The existing literature mainly takes advantage of natural gradients, such as the CO_2 gradients that occur at many volcanic vents worldwide, for example, off the coast of Italy, Papua New Guinea, and Japan [88]. Here, 99% pure CO_2 seeps into the sediments and water column, causing a localized decline in pH compared with sites ~500 m away from the vent, whereas the water chemistry, temperature, light conditions, and other environmental factors stay constant [89]. At the vent sites, the holobiont is chronically exposed to a low pH/elevated CO_2 and is therefore likely adapted or acclimatized to elevated CO_2 conditions. This offers an opportunity to explore the effects of ocean acidification *in situ* and allows for comparisons with control sites, which has been done for other host organisms, such as corals and sponges [89], as well as for macroalgal host dynamics [88], but not yet for the macroalgal holobiont. Gradients for, for example, salinity (near estuaries) and nutrients (e.g., near rivers and aquaculture) can also be found [90]. Seasonal changes in temperature can provide insight into climate-change-related temperature increases [91]. However, these seasonal fluctuations are likely within the lethal boundaries of the macroalgae, and can therefore not fully mimic the effect of climate change. Beyond using geographically specific conditions, the environment can be experimentally manipulated with *in situ* cage experiments. For example, temperature can be locally adjusted using heating elements, incident light can be manipulated by shading areas, and water can be enriched by adding nutrients [92]. Whereas the macroalgae are not long-term adapted in the case of an experimentally manipulated environment, the experiment is still carried out in the complex natural system, as opposed to laboratory studies. And finally, experimentally manipulated hosts can also be used in field experiments, as was demonstrated by Campbell *et al.* [66] who created *Delisea pulchra* thalli unable to produce furanones (its chemical defence) by removing bromine from the culture media. Both furanone-free and furanone-producing algae were transplanted into the field, and the proportion of the thallus that bleached (resulting from infection) was assessed. By using axenic hosts or physiologically modified hosts that are sealed under field conditions within individual flow-through chambers with a specific mesh size, allowing for water and water-borne microbes to pass through while keeping the host inside, it is possible to verify how the holobiont responds to specific environmental conditions in more realistic scenarios. In theory, depending on the mesh size, it could also be possible to prevent microbes from entering the flow-through chamber (e.g., using customized dialysis membranes), but this will likely be expensive, and the membranes may easily clog depending on sedimentation conditions. Although placing these flow-through chambers with the hosts in areas that are subjected to abiotic stress (higher temperature or higher CO_2) or biotic stress (pathogens, other organisms that are competing for space) might provide a more natural, accurate response, it might be difficult to disentangle environmental from geographical effects.

Concluding Remarks and Future Perspectives

It becomes increasingly clear that the microbiota is important for the functioning of macroalgae. Despite this, climate-change-related studies with macroalgae are predominantly focused on the host only, and bacteria are still seen as contamination instead of a crucial component of healthy functioning macroalgae (e.g., seawater is UV filtered to kill microbes). The main question, however, is not how climate change will impact the macroalgal host, but how climate change affects the macroalgal holobiont (see Outstanding Questions). Due to the large amounts of interactions between the environment, the host, and the bacterial communities, it will be very complex to clearly establish causality. However, understanding the mechanisms through which the environment interacts with the holobiont and the magnitude of importance of each component will be essential in understanding the effects of climate change. We believe that the framework and

Outstanding Questions

How are macroalgae impacted by environmental stressors? How are the microbial communities associated with algae impacted by environmental stressors? How are the interactions between macroalgae and microbes affected by environmental stressors?

How do different environmental parameters, combined, influence the holobiont? (i.e., what are the synergistic effects of climate change stressors on the holobiont?)

Does the microbial community differ between healthy and stressed algae? If so, is the difference in health condition of the macroalgae caused by a change in the microbial composition? Or do stressed macroalgae recruit different microbes?

How resistant and how resilient is the macroalgal holobiont to changes in the environment? Is the change in host functioning and microbial community gradual or is there a tipping point? Can the holobiont reach a new stable equilibrium in future ocean conditions? (i.e., can the holobiont adapt?)

What role do microbes play in the defence mechanism of algae against pathogens? Is there a shift to an increased abundance of pathogens associated with algae when the algae are impacted by environmental stress?

So far, research has mainly focused on epiphytic bacteria. Many other components, however, are likely important for the healthy functioning of the holobiont. What role do viruses, fungi, archaea, and protists have in the holobiont? What functional role do endophytic communities have, and in what way do they differ from epiphytic communities?

possible future scenarios provided here will be a valuable tool in classifying the interactions and further help us to understand the effect of climate change on the macroalgal holobiont. We also emphasize the need for manipulative experiments, rather than limiting ourselves to descriptive studies only, as these will allow the establishment of causal relations. There are many different experimental approaches that can help to unravel the seaweed holobiont, and instead of focusing on one approach in particular, we believe them to be complementary. Being able to make use of axenic hosts, however, will be an essential step for many of those approaches. Since macroalgae are a large, diverse, paraphyletic group of organisms, many different holobionts are likely to form, each with a different response to climate change. Similarly, function and taxonomy are not always linked in the microbial world. This highlights the need to start cataloguing genes and their functions of both the microbiome and the host, which will require a combination of metagenomic and metatranscriptomic studies, linking microbial and host gene expression. Applying such a systems biology approach may be the first step in deciphering the complex interactions between the environment and all the components that together form the macroalgal holobiont. Finally, the holobiont may be a functional unity, it is not an isolated unity. While the microbiota alters how the host has access to its environment, the functional outcome of a holobiont will, in turn, also affect the surrounding organisms, with major potential effects on ecosystem and economy.

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