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MOLECULAR AND FUNCTIONAL ECOLOGY OF AQUATIC MICROBIAL SYMBIONTS

Topic Editors
Hans-Peter Grossart, Lasse Riemann, and Kam W. Tang

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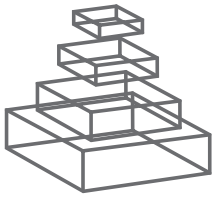

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MOLECULAR AND FUNCTIONAL ECOLOGY OF AQUATIC MICROBIAL SYMBIONTS

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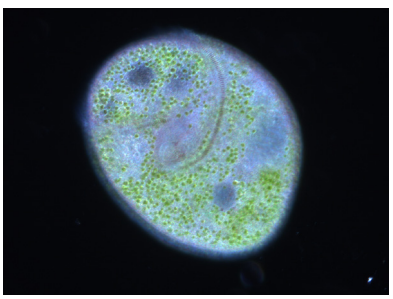

The cilliate Stentor amethystinus is a perfect model system for studying interactions between microorganisms and higher organisms. It harbors a number of microbial endosymbionts, i.e. photoautotrophic (green algae and cyanobacteria) and heterotrophic bacteria (including a high diversity of Bacteria and Archaea). The dark-field microscopy image shows S. amesthystinus from Lake Stechlin, the length of the organism is ca. 1 mm.

Aquatic organisms are densely colonized by a wide variety of microorganisms, such as bacteria, fungi, algae, and protozoans. For example, a rough estimate based on literature data suggests that the abundance of zooplankton-associated bacteria alone can rival or exceed that of free-living bacteria. With the exception of a few pathogens, little is known about the ecology of these microbial symbionts, such as their life cycle, their interactions with the hosts and adjacent microbes, and the evolution of this symbiotic relationship. Higher organisms present specific microhabitats with very different environmental conditions than the surrounding water, and they may therefore support the proliferation and activities of distinct microbial communities, with important biogeochemical consequences. For example, earlier research has suggested that the guts and feces of zooplankton and fish may support anaerobic microbial processes that otherwise cannot occur in the oxygen-rich water columns.

Recent advances in methodology such as microprofiling allow researchers to characterize microhabitats within the higher organisms in unprecedented details. Rapid development of single-cell and molecular techniques for phylogenetic and physiological analyses also offers enormous opportunities to study these symbionts at different scales, from a single gene to the whole community, and even their evolutionary changes in near real time. New experimental approaches using genetically accessible model systems and individual-based modeling can also provide a mechanistic and systematic understanding of host-symbiont relationships.

This special issue aims to highlight new findings on the molecular and functional ecology of microbial symbionts in all aquatic environments. The goal is to promote exchange among experts from various fields to advance a conceptual framework for future studies on the interactions between microbial symbionts and higher organisms in aquatic systems.

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EDITORIAL
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Molecular and functional ecology of aquatic microbial symbionts

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Traditional aquatic microbial ecology has largely neglected organism-associated microorganisms in biodiversity and ecosystem function studies. Living aquatic organisms provide habitats for a wide variety of microorganisms, such as bacteria, fungi, algae, and protozoans. A rough estimate based on literature data indicates that bacteria densely colonize algae and zooplankton, reaching densities (i.e., number of bacteria per unit biovolume) far higher than in the ambient water. The relationship between these microbes and the base organisms can range from commensalism, parasitism to mutualism. With the exception of a few pathogens, surprisingly little is known about the ecology of microbial symbionts, such as their life cycle, their interactions with the hosts and adjacent microbes, and the evolution of such symbiotic relationships. However, recent whole genome sequence data suggest that symbiotic bacteria contribute substantially to the functional biodiversity in the aquatic world, influence the fitness of the host organisms, and thereby ecosystem functioning. Microhabitats within the higher organisms provide very different environmental conditions than the surrounding water, and they may therefore support the proliferation and activities of distinct microbial communities with important biogeochemical consequences. For example, earlier research suggested that the guts and feces of zooplankton and fish may support anaerobic microbial processes that otherwise cannot occur in oxygen-rich waters.

Recent advances in methodology such as profiling using microsensors allow researchers to characterize microhabitats within the higher organisms in unprecedented detail. Rapid development of single-cell and molecular techniques for phylogenetic and physiological analyses also offers enormous opportunities to study these symbionts at scales from a single gene to the whole community, and even their evolutionary history. New experimental approaches using genetically accessible model systems and individual-based modeling can also provide a mechanistic understanding of host-symbiont relationships.

This special issue brings together 11 articles that highlight new findings on biodiversity and functions of aquatic microbial symbionts, including microbial assemblages in close association with higher organisms.

The article by Wahl et al. (2012) presents a conceptual framework for studying the role of bacteria on the outer body of marine organisms, which represents a highly active interface between host and biofilm microbes. The authors show that biodiversity and functions of the attached microbiota are largely dependent on environmental parameters, and how the microbiota influences the host's ecology and health. The article by Bickel et al. (2012) focuses on ciliate epibionts of crustacean zooplankton in lakes. The authors show that ciliate epibiont abundance varies greatly between lakes and zooplankton species, respectively. Although the ciliate epibionts exhibited high grazing rates on free-living bacteria, their effects on the total bacterial abundance seemed to be rather low. Also, effects of epibionts on the physiology and development of their host require further study.

A cluster of three articles (Dziallas et al., 2012; Garcias-Bonet et al., 2012; McManus et al., 2012) addresses endosymbiotic relationships between microbes and higher organisms. The article by Dziallas et al. (2012) reviews knowledge on the diversity, ecological function, and evolution of ciliate symbionts and also points at several future research directions. The article by McManus et al. (2012) more specifically addresses chloroplast symbioses in marine ciliates. The authors discuss benefits and costs of chloroplast symbiosis for the ciliate *Strombidium rassoulzadegani*, and for the photo- and feeding-physiology of the ciliate. The paper by Garcias-Bonet et al. (2012) evaluates the biodiversity of endophytic bacterial communities on a marine angiosperm in the Mediterranean. The presence of bacterial endophytes greatly differed among locations and tissue types indicating a highly specialized bacterial endophyte community with potentially diverse and important functions for the angiosperm host.

Parasitic fungi in phytoplankton and parasitic dinoflagellates in marine copepods are the research foci in the papers by Sime-Ngando (2012) and Skovgaard et al. (2012), respectively. Both papers highlight the importance of parasites for plankton ecology and biodiversity, and planktonic food web dynamics. Several lines of evidence indicate that parasites have the potential to drive genetic diversity at the species, population, and community levels of pelagic ecosystems.

The next two articles evaluate the role of symbionts of an ascidian (Kühl et al., 2012) and a coral (Wangpraseurt et al., 2012) in photosynthetic activities in relation to environmental parameters such as irradiation, periods of anoxia, and physiology of the host. Kühl et al. (2012) focus on a chlorophyll *b*-containing

symbiotic cyanobacterium and its photosynthetic activity, whereas Wangpraseurt et al. (2012) examine optical microniches in corals and provide evidence for the importance of such microniches for photobiology and stress response of the corals, as well as for the phenotypic and genotypic plasticity of the coral symbionts.

Finally, the articles by Dinasquet et al. (2012) and Rivera et al. (2013) report on the contributions of organism-associated bacteria to spatial heterogeneity of bacterioplankton activity and community composition in the sea. Dinasquet et al. (2012) show data indicating specific associations between certain bacterioplankton groups and their jellyfish host, whereas Rivera et al. (2013) highlight the role of ballast water for the dispersal of potentially pathogenic vibrio species.

In summary, these articles cover a range of symbiotic relationships in aquatic environments, pinpoint that these relationships are widespread, and that they conceivably play important roles for the health, adaptation, and evolution of the host organisms, and, thereby, for food web structure and ecosystem functioning. We hope that this special issue will stimulate more discussions and research on the fascinating subject of microbial symbiosis, which seems to be the rule rather than the exception in aquatic ecosystems.

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The second skin: ecological role of epibiotic biofilms on marine organisms

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In the aquatic environment, biofilms on solid surfaces are omnipresent. The outer body surface of marine organisms often represents a highly active interface between host and biofilm. Since biofilms on living surfaces have the capacity to affect the fluxes of information, energy, and matter across the host's body surface, they have an important ecological potential to modulate the abiotic and biotic interactions of the host. Here we review existing evidence how marine epibiotic biofilms affect their hosts' ecology by altering the properties of and processes across its outer surfaces. Biofilms have a huge potential to reduce its host's access to light, gases, and/or nutrients and modulate the host's interaction with further foulers, consumers, or pathogens. These effects of epibiotic biofilms may intensely interact with environmental conditions. The quality of a biofilm's impact on the host may vary from detrimental to beneficial according to the identity of the epibiotic partners, the type of interaction considered, and prevailing environmental conditions. The review concludes with some unresolved but important questions and future perspectives.

Keywords: stress, microbe-macroorganism interaction, modulation of interactions, epibiosis, chemical ecology, biofilm

MICROBIAL COMMUNITIES ASSOCIATED WITH MACROORGANISMS IN THE SEA: ANTAGONISM, NEUTRALISM, SYNERGISM IN EPIBIOSIS

In contrast to air, the ocean represents a benign environment for most living organisms: With the exception of some harsh marine environments, the means of physico-chemical properties are generally not far off the optimum of most species and their fluctuations are moderate, rarely exceeding biological tolerance limits. As a consequence, insulating coatings of the epidermis such as hair, feathers, wax are not required in the marine realm. When protective armor against predation or mechanical stress (cuticles, shells, spines, tunics, etc.) is not realized because a species rather relies on escape, hiding, poor palatability or chemical defenses, its outer body surface represents its major physiological interface with the environment. This interface is often delicate serving a multitude of exchange processes with the environment: respiration, exudation of wastes and secondary metabolites, absorption of energetic irradiation or informational signals, uptake of nutrients, and gases, etc. The body surface of a nudibranch, for instance, may be considered the combined equivalent of human skin, eyes, (internalized) lungs, intestine, and kidneys. From an ecological perspective, most interactions among conspecifics, or host/parasite and predator/pray pairs are linked to and controlled by properties of the organism's body surface. Finally, most environmental stressors such as, e.g., desalination, hypoxia, UV radiation, and pollution are experienced at this functional interface foremost.

The functioning of such delicate interfaces is threatened by fouling, i.e. the settlement of other organisms onto this surface. Such non-trophic association between a basibiont (host) and an epibiont (on-growing organism) is called epibiosis (e.g., Wahl, 1989). The dispersion stages of potential settlers, ranging from bacteria to the propagules of invertebrates or macroalgae are always present in the sea albeit varying in composition and concentration regionally, with depth, and with season. The concentration of the various forms can reach densities per ml of seawater of 10^6 for bacteria, 10^3 for microalgae, and 10^2 for propagules of animals and macroalgae (for references, see Harder, 2009). It is not surprising therefore that any undefended surface is overgrown by micro- and macro-foulers within days or weeks. Such an uncontrolled biotic coverage of an organism's body surface will have a multitude of, mostly detrimental, consequences for the basibiont: Increased weight and friction, impeded trans-epidermal exchanges, altered color, smell, and contour with multiple consequences. These proximate changes to the host due to epibiosis may lead to a loss of buoyancy, an impediment of motility, a hindrance to mating, or a substantial shift of interactions among species (e.g., Prescott, 1990; Dougherty and Russell, 2005; Wahl, 2008b) and is thought to be the selecting force behind the evolution of a variety of antifouling strategies. While the direct and indirect effects of macro-epibiosis, i.e., the colonization of a basibiont by macroscopic epibionts have been thoroughly studied, the consequences of epibiotic microbial fouling have received substantially less attention. The reasons for this asymmetry of investigative effort are obvious: The presence of epibiotic biofilms (microbes enclosed within an exopolymeric matrix) is less conspicuous, its constituents are not comprehensively described (for the most part, the constituents are inaccessible by common culture techniques and even the recent advent of molecular tools and fast sequencing techniques does not lead

to identification of all organisms in the biofilm), and its abundance and compositions seem to be highly variable and dynamic. Furthermore, it is difficult to study the functioning of epibiotic biofilms without the confounding input of the host. Finally, due to their thinness and often negligible biomass the physiological and ecological impact of epibiotic biofilms until recently may have been severely underestimated.

Biofilms develop easily at any solid/liquid interface in humid or aqueous environments. By a dynamic equilibrium between settlement of planktonic ("free") bacteria and detachment of biofilm bacteria the two major bacterial compartments remain connected (Grossart, 2010). Free bacteria are attracted to point sources of organic matter, such as aggregates or organism surfaces rapidly react to appropriate stimuli by attachment and physiological shifts (Grossart and Tang, 2010). These authors describe that biofilm bacteria in comparison to their planktonic life form, are more densely packed by 1–2 orders of magnitude (**Figure 1**), communicate more intensively, show higher enzymatic activity, growth and production, and exercise more intense lateral gene transfer. At the same time they seem to be more susceptible to predation and infection in the attached life stage. Bacterioplankton is well studied in most regards while knowledge about the biology and ecology of biofilm bacteria is just emerging. Early studies on the role of biofilms stem mostly from the medical and the technical fields. Composition and functioning of biofilms have been thoroughly investigated on internal (and external) surfaces of the human body (plaque, intestinal flora, bacterial fouling on implants: reviewed by Robinson et al., 2010) and on technical surfaces as diverse as sensor heads, reverse osmosis membranes of desalination plants, drink water pipes, or ship hull paints (reviewed in Dürr and Thomason, 2010 and references therein; Railkin, 2004; Flemming, 2009). Apart from clogging, shading, corrosive, and degrading effects, the major interest of many researchers was the fouling-mediating role of biofilms (reviews by Dobretsov et al., 2009; Hadfield, 2011). The consensus of most investigations is that the presence of biofilms alters the substratum physically (wettability, microtopography, consistency) and chemically (alteration of the substratum, degradation of substances released by the substratum, exudation of bacterial metabolic products), and that they have the capacity to modulate (reduce, enhance, select) the recruitment of other bacteria, diatoms, fungi, larvae, or spores. The capacity to hinder further fouling seems to be more prevalent in epibiotic biofilms than in the bacterial assemblage of the water column (Burgess et al., 1999). Whether this is also true for the enhancement of settlement (Hadfield, 2011) is not known. However, the establishment of epibiosis is not a simple process, and various physical and chemical properties of the host surface, as well as interactions among the settlers are determinants of the formation of specific communities (e.g., Wahl et al., 2010; Steinberg et al., 2011).

It is obvious that the multiple possible functions and activities of biofilms (described in later sections and depicted in **Figure 2**) render their presence on living surfaces everything but trivial. There are probably no marine organisms whose surface is free of epibiotic bacteria and only very few continuously exhibit an almost sterile surface such as some colonial didemnid ascidians (Wahl and Lafargue, 1990). The vast majority of marine organisms bear epibiotic biofilms of variable density and composition (e.g., Lachnit et al., 2009; Grossart, 2010). Considering the diversity of the already known effects, it can be expected that the nature of this biofilm will affect the basibiont's physiology and ecology in beneficial, detrimental, or ambiguous ways. In fact, since biofilms in form and function are considered almost analogous to multicellular organisms (Steinberg et al., 2011) epibiotic microfouling leads to the replacement of the host's epidermis as the sole functional interface between host and environment by a new, and functionally different, "tissue," the epibiotic biofilm. Cells in this biofilm "tissue" interact with each other, exchange metabolites and information, multiply and even produce propagules ("dispersers") when internal or external conditions degrade (reviewed in Steinberg et al., 2011). The analogy to multicellular organisms, however, is limited by the facts that cells in multispecies biofilms do not share the same genome and that each establishment of a biofilm produces a differently composed "organism" albeit with often similar functionality (Burke et al., 2011a). The following review will give evidence of our still embryonic knowledge on the ecological role of biofilms epibiotic on marine organisms. In this review, we focus on effects the host experiences from this association with a biofilm while being well aware that the interaction is reciprocal and biofilm bacteria are affected by host traits in many regards.

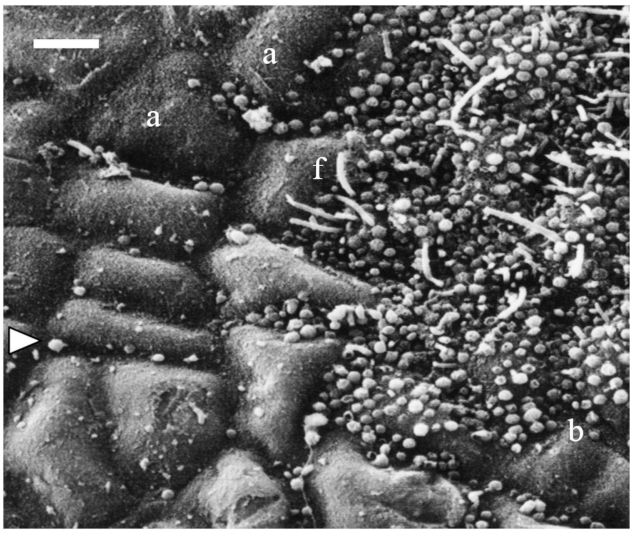

FIGURE 1 | Scanning electron micrograph showing a partially fouled surface of *Fucus vesiculosus* with unobstructed and masked areas of host tissue. The left side of the picture shows an apparently clean surface, the algal cells are visible (a) and also few coccoid bacteria (arrow) between them. In contrast, the right side of the picture shows a microbial film with coccoid bacteria (b) and filaments (f) covering the algal cuticle. The photo also illustrates the patchiness of microfouling on one host individual. Scale bar = 5 μm.

BACTERIAL COMMUNITIES AT THE SURFACE OF MACROORGANISMS

In nature, every single macroorganism is found to maintain more or less stable relationships with prokaryotes (McFall-Ngai, 2000, **Table 1** for algal hosts). Some core roles of bacteria for the development and evolution of the host have recently been

Table 1 | Phylogenetic studies of the bacterial communities associated with macroalgae.

Algal species	Molecular technique	Bacterial phyla	Country origin	Reference
CHLOROPHYTA				
Bryopsis hypnoides	CLO, FISH	BA, FI, PR (al, ep, ga)	Mexico	Hollants et al. (2011a,b)
Bryopsis pennata	CLO, FISH	BA, FI, PR (al, ep, ga)	Mexico	Hollants et al. (2011a,b)
Caulerpa taxifolia	CLO, RFLP	BA, **PR** (**al**, be, de, ga)	4 Countries	Meusnier et al. (2001)
Chara aspera	FISH	AC, **BA**, PL, **PR** (**al**, be, ga)	Germany	Hempel et al. (2008)
Desmidium grevillii	CLO	BA, PR (al, be, ga)	USA	Fisher et al. (1998)
Halimeda opuntia	CLO	AC, BA, CH, CL, CY, FI, PL, PR	N. Antilles	Barott et al. (2011)
Hyalotheca dissiliens	CLO	BA, PR (al, be, ga)	USA	Fisher et al. (1998)
Spondylosium pulchrum	CLO	BA, PR (al, be, ga)	USA	Fisher et al. (1998)
Ulva australis	CLO, DGGE	AC, **BA**, PL, **PR** (al, de, **ga**)	Australia	Longford et al. (2007)
Ulva australis	CLO	AC, **BA**, CY, PL, **PR** (**al**, de, ga), VE	Australia	Burke et al. (2011b)
Ulva australis	CFISH, DGGE	BA, **PR** (**al**, ga)	Australia	Tujula et al. (2010)
Ulva intestinalis	CLO, DGGE	AC, BA, **PR** (**al**, de, ep, ga), VE	Germany	Lachnit et al. (2011)
Ulva prolifera	CLO, DGGE	AC, BA, CY, FI, FU, PL, **PR** (**al**, be, de, ep, **ga**), SP, VE	China	Liu et al. (2011)
HETEROKONTOPHYTA				
Dictyota bartayresiana	CLO	AC, BA, CH, CL, CY, FI, PL, PR	N. Antilles	Barott et al. (2011)
Fucus vesiculosus	CLO, DGGE	**BA**, **CY**, PL, **PR** (**al**, be, de, ep, **ga**), **VE**	Germany	Lachnit et al. (2011)
Laminaria hyperborea	DGGE, FISH	AC, BA, CY, **PL**, **PR** (**al**, be, ga), VE	Norway	Bengtsson et al. (2010)
Laminaria rodriguezii	CLO	AR*, PR (be)	Spain	Trias et al. (2012)
Saccharina latissima	CLO, DGGE	BA, **PR** (**al**, **ga**)	Germany	Staufenberger et al. (2008)
RHODOPHYTA				
Coralline crustose	CLO	AC, BA, CH, CL, CY, FI, PL, PR	N. Antilles	Barott et al. (2011)
Delisea pulchra	CLO, DGGE	AC, BA, CH, CY, PL, **PR** (**al**, de, **ga**), VE	Australia	Longford et al. (2007)
Delisea pulchra	CLO, DGGE	BA, PL, **PR** (**al**, ga)	Australia	Fernandes (2011)
Gracilaria vermiculophylla	CLO, DGGE	AC, CY, DT, PL, **PR** (**al**, be, de)	Germany	Lachnit et al. (2011)
Osmundaria volubilis	CLO	AR*, PR (be)	Spain	Trias et al. (2012)
Phyllophora crispa	CLO	AR*, PR (be)	Spain	Trias et al. (2012)
Porphyra yezoensis	CLO	**BA**, LE, **PR** (al, be, **ga**)	Japan	Namba et al. (2010)
3 spp. macroalgae	CLO, TRFLP	**BA**, CY, PL, PR (al, ga), VE	Chile	Hengst et al. (2010)
12 spp. macroalgae	CLO	PL	Portugal	Lage and Bondoso (2011)
Unidentified turf algae	CLO	AC, BA, CH, CL, CY, FI, PL, PR	N. Antilles	Barott et al. (2011)

The techniques utilized by the different authors for analyzing the microbial communities of brown (Heterokontophyta), green (Chlorophyta), and red (Rhodophyta) macroalgae are denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), sequencing of 16S rRNA gene libraries (CLO), and terminal restriction fragment length polymorphism (TRFLP).

The bacterial phyla are represented by Actinobacteria (AC), Bacteroidetes (BA), Chlorobi (CL), Chloroflexi (CH), Cyanobacteria (CY), Deinococcus-Thermus (DT), Firmicutes (FI), Fusobacteria (FU), Lentisphaerae (LE), Planctomycetes (PL), Proteobacteria (PR) from which belong the bacterial classes Alpha-proteobacteria (al), Betaproteobacteria (be), Delta-proteobacteria (de), Epsilon-proteobacteria (ep) and Gammaproteobacteria (ga), Spirochaetes (SP), and the phylum Verrucomicrobia (VE). Also members of the Archaea (AR) are considered. In bold are represented the dominant groups (when quantified).*

reviewed (Fraune and Bosch, 2010). Most bacteria, and particularly those associated with the surface of other organisms, occur in biofilms (Steinberg et al., 2011). Biofilms on the surface of marine organisms are usually dominated by prokaryotes (Bacteria), while eukaryotes such as diatoms, fungi, and protozoa can be present at lower abundance (Bodammer and Sawyer, 1981; Höller et al., 2000; Burja and Hill, 2001; Hentschel et al., 2003; Webster and Taylor, 2012). Usually, the ratio bacteria:diatoms:flagellates in biofilms is 640:4:1 (Railkin, 2004). On undefended surfaces in temporal waters, bacterial densities typically reach densities of 10^7 cm^{-2} or higher within a couple of weeks (Railkin, 2004 and references therein, Jones et al., 2008). The densities of epibiotic bacteria can vary substantially, depending on the species and their physiological status of the host but are typically lower. While the surfaces of some crustaceans such as the decorator crabs are heavily colonized (Hultgren and Stachowicz, 2011), surfaces of colonial didemnid tunicates remain almost free from microbes (Wahl and Lafargue, 1990). The abundance of the epibiotic bacterium *Pseudoalteromonas tunicata* on marine eukaryotic hosts is 3–4 orders of magnitude lower than on inert substrata ($<1 \times 10^3$ cells cm^{-2}: Skovhus et al., 2004). Similarly, the densities of bacteria on soft corals were found to be low (about 5 to 10×10^3 cells cm^{-2}; Harder et al., 2003), cell densities which were similar to those on the alga *Caulerpa racemosa* (about 20×10^3 cells cm^{-2}; Dobretsov et al., 2006a). Densities of bacteria on the surface of the alga *Ulva reticulata* also were 2.3-fold lower than on undefended glass surfaces

(about 27×10^3 cells cm^{-2}; Dobretsov and Qian, 2002). Densities of bacteria on the sponge *Haliclona* sp. were twofolds higher than on neighboring inanimate substrata, while those on the sponges *H. cymaeformis* and *Callyspongia* sp. were significantly lower (about 13 to 20×10^3 cells cm^{-2}; Dobretsov et al., 2005). The macroalga *Laminaria hyperborea* shows very variable cell densities in its biofilm (8.3×10^2 to 6.3×10^7 cm^2: Bengtsson et al., 2010) while *Fucus vesiculosus* overall exhibits a more dense biofilm (7.7×10^6 to 1.9×10^8: Wahl et al., 2010).

Severe reduction of biofilm density relative to undefended surfaces and a specificity of their taxonomic composition (as treated below) indicate an active (pro-, antifouling) or passive (surface properties, exudates) role of the hosts in the recruitment of epibiotic bacteria.

BIOFILMS ON ALGAE

Algae are a phylogenetically and morphologically extremely diverse group. They can be uni- to multicellular and from few μm to many m long. Although unicellular microalgae are subject to bacterial settlement (e.g., Grossart, 2010), structured microbial communities such as multispecies 3-D biofilms rarely develop on their surfaces (for references, see Follows and Dutkiewicz, 2011). This contrasts with multicellular ("macro-") algae, which are especially susceptible to epibiosis and are typically covered by diverse microbial communities which may include bacteria, microalgae, fungi, and protists (Lobban and Harrison, 2000; Kohlmeyer and Volkmann-Kohlmeyer, 2003). Bacteria, typically by far the most abundant epibionts (see above), play a key role in the colonization and biofouling processes on macroalgae (Corre and Prieur, 1990): Algal tissue represents a rich source of organic nutrients which are a cue for some bacteria (e.g., Grossart, 2010). Since bacteria are omnipresent in the water column year-round, have a small reaction time, are highly adaptive and capable of rapid metabolization of algal exudates they are likely to be early colonizers (Fernandes, 2011), starting the biofilm process (e.g., Wahl, 1989; Goecke et al., 2010).

There is growing evidence that the composition of bacterial communities on the surface of macroalgae differs from that in the surrounding seawater or on inanimate (and undefended) substrata in close vicinity (e.g., Dobretsov et al., 2006a; Staufenberger et al., 2008; Lachnit et al., 2009; Bengtsson et al., 2010; Burke et al., 2011b). Comprehensive phylogenetic assessments of whole bacterial communities on algal surfaces are still scarce (but see Burke et al., 2011b). However, data of molecular studies (**Table 1**) – supported by culture-based studies (reviewed by Goecke et al., 2010) – are emerging and begin to provide important insights into the dynamic associations between macroalgae and bacteria. 16S rRNA gene sequences retrieved from epiphytic bacteria on freshwater and marine macroalgae belong to several major lineages within bacteria: Alpha, Beta, and Gamma classes of the Proteobacteria, the Bacteroidetes, and the Actinobacteria (Fisher et al., 1998; Longford et al., 2007; Hempel et al., 2008, see **Table 1**). At a higher taxonomic level, the microbial groups that dominate surface communities on macroalgae – Proteobacteria and Bacteroidetes – are the same as in most aquatic environments (Cottrell and Kirchman, 2000; Sapp et al., 2007). A prevalence of sequences from these two bacterial phyla has been detected in phytoplankton (Riemann et al., 2000; Schäfer et al., 2002), and on green algae (Meusnier et al., 2001; Longford et al., 2007; Hempel et al., 2008; Burke et al., 2011b; Hollants et al., 2011a; Liu et al., 2011), brown algae (Staufenberger et al., 2008; Wiese et al., 2009; Bengtsson et al., 2010; Lachnit et al., 2011), and red algae (Namba et al., 2010; Fernandes, 2011; Lachnit et al., 2011), but also on invertebrates (Tait et al., 2007; Mangano et al., 2009, among others), suggesting that those marine bacteria are common and – possibly – important micro-epibionts on many different organisms. Especially the *Roseobacter* clade of the Alpha-proteobacteria has been identified as one of the most prevalent groups in the bacterial assemblages associated with phytoplankton (Schäfer et al., 2002; Seyedsayamdost et al., 2011) and macroalgae from different geographical locations (Staufenberger et al., 2008; Hengst et al., 2010; Namba et al., 2010; Tujula et al., 2010; Fernandes, 2011; Liu et al., 2011). Other phyla, such as the Planctomycetes, Verrucomicrobia, and Cyanobacteria (Bengtsson et al., 2010; Lachnit et al., 2011; Lage and Bondoso, 2011) have just recently been recognized as frequent colonizers of macroalgal surfaces. Members of the Firmicutes are frequently found among the cultivatable bacteria associated with macroalgae and are usually also relatively prominent among the total bacteria that are identified in molecular studies (Wiese et al., 2009; Goecke et al., 2010). Other phyla detected less frequently on the surfaces of macroalgae are Chlorobi, Chloroflexi, Deinococcus-Thermus, Delta-proteobacteria and Epsilon-proteobacteria, Fusobacteria, Lentisphaerae, and Spirochaetes (but, see Barott et al., 2011; Liu et al., 2011, **Table 1**). Furthermore, members of the domain Archaea have been very recently detected on macroalgae in mesophotic depth in Spain (Trias et al., 2012).

This high diversity of bacterial epibionts is not randomly distributed among algal host species. More recent research confirms that different species of marine macroalgae in the same habitat support differently composed bacterial communities (Lachnit et al., 2009, 2011; Nylund et al., 2010; Trias et al., 2012), while specimens of the same algal even in different environments tend to be associated with highly similar bacterial communities (Staufenberger et al., 2008; Lachnit et al., 2009). The relationship between environmental factors and non-epibiotic bacterial abundance and community composition has been well documented in various marine ecosystems (Sapp et al., 2007). Even on two conspecific host individuals a complete overlap in the epibiotic microbial communities cannot be expected, because aquatic systems are usually subject to drastic spatial, temporal (seasonal) and post-disturbance shifts (Corre and Prieur, 1990; Longford et al., 2007; Staufenberger et al., 2008; Fernandes, 2011; Liu et al., 2011), and the physiological state of the host (age, senescence, diseases) may affect the associated bacterial community via exuded metabolites (Goecke et al., 2010; Seyedsayamdost et al., 2011).

BIOFILMS ON ANIMALS

Recent studies suggested that surfaces of most invertebrates and vertebrates stay relatively free from macrofouling while they usually feature some degree of microbial fouling (Richmond and Seed, 1991; Dobretsov et al., 2006b). Both, culture dependent and polymerase chain reaction (PCR) based studies have revealed that microorganisms associated with animals differ from those in the water column and those associated with other types of substrata

in the neighborhood, suggesting that these associations are specific to some degree (Burja and Hill, 2001; Harder et al., 2003; Hentschel et al., 2003; Lee and Qian, 2003; Thakur et al., 2004; Qian et al., 2006; Webster and Taylor, 2012). Most of these studies are based on investigation of sponge – associated endosymbiotic microorganisms, while the information about microbes internally or externally associated with other animals is limited (but, see: Bodammer and Sawyer, 1981; Pukall et al., 2001; Harder et al., 2003; Kittelmann and Harder, 2005; Perez-Matos et al., 2007; Winters et al., 2010). It has been demonstrated that the community composition of epibiotic bacteria associated with the same sponge species from different locations remained consistent (Lee et al., 2006a, 2011), while microbial communities associated with different species of sponges differed substantially (Qian et al., 2006; Lee et al., 2011). This suggests a certain host-specificity of the biofilms (as it has been shown in algae) while in the vast majority of cases a mandatory restriction of a given bacterial strain to a particular host species has not yet been shown.

The composition of epibiotic bacterial communities associated with marine organisms is influenced by temporal changes in the environment (Thakur et al., 2004; Lee et al., 2006b). However, some particular bacteria are specifically and persistently associated with particular marine animals and not present in seawater or on other animals (Thakur et al., 2004; Sharp et al., 2007). For example, *Candidatus Endobugula sertula* is specifically associated with the surface of bryozoan larvae *Bugula neritina* and protects them from predatory fishes (Sharp et al., 2007). Another bacterium – *Bacillus* sp. – was always and exclusively associated with surfaces of the sponge *Ircinia fusca* (Thakur et al., 2004). Besides few cases (see Gustafson and Reid, 1988), it is uncertain whether specific animal symbionts are transmitted vertically via gametes or larvae from adults. The study by Sharp et al. (2007) demonstrated that the mass spawning corals *Montastraea annularis*, *M. franksi*, *M. faveolata*, *Acropora palmata*, *A. cervicornis*, *Diploria strigosa*, and *A. humilis* do not transmit their epibiotic bacteria via their gametes, and bacteria colonize corals only after their settlement and metamorphosis. This suggests that interactions between juvenile forms and epibiotic bacteria are particularly important for the formation of host-specific assemblages of bacteria.

The density of epibiotic bacteria on animal surfaces varies enormously at numerous scales from within-individual to among species, habitats, regions, and seasons (see references cited in the previous paragraph). Some didemnid ascidians exhibit an almost sterile surface with 0 to 1.5×10^2 cells cm^{-2} (Wahl and Lafargue, 1990). Epibacterial densities on sponge surfaces range from almost sterile (60 cells cm^2: *Crambe* crambe,), over strongly reduced (3 to 4×10^4 cells cm^{-2}: *Ircinia fasciculata*, *Spongia officinalis*, Becerro et al., 1994) to "normally fouled" (6.93×10^6 cells cm^2: *Ceratoporella nicholsoni*, Santavy et al., 1990; 7 to 15×10^6 *Ircinia ramosa*, Thakur and Anil, 2000). Corals may have low (5×10^5 cells cm^{-2}: various species, Koh, 1997) or remarkably high densities of epibiotic bacteria (8.3×10^8 cells cm^{-2}: *Oculina patagonica*; Koren and Rosenberg, 2006). The bacterial densities on the carapaces of a variety of crustaceans ranged between 7×10^4 and 3×10^6 cells cm^{-2}, Becker, 1996). The bryozoan *Conopeum reticulatum* features 5×10^7 cells cm^{-2} on its surface (Kittelmann and Harder, 2005). When the bacterial density in the epibiotic biofilm is substantially reduced relative to neighboring species or inanimate substrate in the habitat, the host surface apparently is unsuitable for settlement and/or growth of bacteria due either to physiological exchange processes through the epidermis (e.g., extreme pH fluctuations during diurnal switches between photosynthesis and respiration) or to the deployment of defensive secondary metabolites. However, antimicrofouling mechanisms of the host are not subject of this review.

INTERNAL ASSOCIATIONS

Although not the prime focus of this review, internal association will be briefly treated here because they frequently derive from epibiotic biofilms. Certain types of bacteria have been able to penetrate the host tissue and even overcome the cell membrane and develop an obligatory dependence between bacteria and host (see Woyke et al., 2006; Thornhill et al., 2008; Hollants et al., 2011a). Such endosymbioses with prokaryotes have been established multiple times in many of the major metazoan groups and the diversity of these associations demonstrates their plasticity and evolutionary success (Dubilier et al., 1999; McFall-Ngai, 2000). This is not surprising because many symbionts have an important, mostly beneficial effect on their host, although pathogenic and saprophytic relationships are also involved (Sipe et al., 2000; Woyke et al., 2006; Goecke et al., 2010). The transmission of endosymbionts proceeds in one of three ways: By vertical transmission (transfer from parent to offspring), by horizontal transmission (involving the spread of symbionts between neighboring hosts), or by reinfection of the new host generation from the environmental stock of microorganisms (see Gustafson and Reid, 1988). The bacterial symbiont is not a passive player in the colonization process (McFall-Ngai, 2000). Both, the horizontal transmission of endosymbionts or the reinfection of the new host generation from an environmental stock of microorganisms are likely to involve contact with the host's biofilm. This was proven for, e.g., *Vibrio fischeri* which colonizes the light organs of squid only after specific contact based on both, host ciliar structures and bacterial cell wall components to the juveniles (Visick and Ruby, 2006). Unfortunately, the cultivation of those microbial consortia in the absence of their host is hindered by severe technical difficulties (McFall-Ngai, 2000), which are a barrier to the further elucidation of their biological roles (Moss et al., 2003) and competition during the recolonization of the host's offspring.

ECOLOGICAL ROLE OF EPIBIOTIC BACTERIA: MODULATION OF HOST-ENVIRONMENT INTERACTIONS

The recent increase in studies of the phylogenetic diversity of bacterial communities associated with marine organisms starts to provide information on the presence and absence of specific taxa under various environmental conditions and on different hosts. However, it provides little information on the ecological function of these taxa. The *in situ* functioning of epibiotic strains or communities is difficult to study. A new and promising approach is metagenomic sequence analysis, which was used to investigate the relation between community structure and community function in the bacterial assemblages associated with *Ulva australis* (Burke et al., 2011b). Despite a high phylogenetic variability in the

microbial species composition the authors discovered only little functional variability (measured as presence of functional gene clusters). Phylogenetically different bacterial species (or strains) of the regional/seasonal colonizer pool – able to colonize one particular host species – that can carry out similar metabolic and other functions apparently compete with each other in the colonization of algal surfaces (Burke et al., 2011a; Fernandes, 2011). Due to remarkable functional redundancy structural differences in the epibiotic biofilm are not necessarily associated with a shift in function. Since for the host and its interactions with the environment biofilm function matters more than phylogenetic biofilm composition, investigations at the functional level based on genomic or metabolomic information should become more prominent in the future.

The composition and metabolism of a biofilm have the capacity to substantially modulate the interactions of the host with its living and non-living environment (see below). Both traits of the biofilm are affected by host properties (not treated here), environmental conditions and interactions within the biofilm. The complex architecture of a mature biofilm provides niches with distinct physico-chemical conditions, differing, e.g., in oxygen availability, in concentration of diffusible substrates and metabolic side products, in pH, and in the cell density (Costerton et al., 1999). In such a mixed microbial community the strains may interact antagonistically or synergistically with each other, the latter resulting in co-colonization of distinct groups of bacteria having metabolic cooperation (Kuchma and O'Toole, 2000; Andersson et al., 2008; Nadell et al., 2009). Microbial processes such as nitrification, anaerobic degradation of organic compounds, or bioremediation of xenobiotic compounds, have been shown to require interactions between different bacterial species within the biofilm (Paerl and Pinckney, 1996). This metabolic cooperation is advantageous to the micro-community. Nevertheless, cooperation among species is only expected under restricted conditions (Nadell et al., 2009). Under natural conditions, bacteria compete (intra- or inter-specifically) intensely with their neighbors for space and resources. A surface (especially of hosts) may itself also be a trophic source where attached microorganisms catabolize organic or inorganic nutrients directly (Madigan and Martinko, 2006; Grossart, 2010). Therefore, the presence of other microorganisms on a surface reduces the availability of substrate and substratum for colonizing species (Prado and Kerr, 2008).

Under such competitive selection it is not surprising that bacteria have developed special mechanisms in order to interfere with the capability of other antagonistic bacteria during the process of surface colonization and acquisition of nutrients (Falagas et al., 2008). The mechanisms of bacterial antagonism would include depletion of some essential substances (e.g., a substrate or a vitamin), alteration in the microenvironment (e.g., changes in the gas concentration or pH), or production of an antagonistic substance (e.g., antibiotics; Wannamaker, 1980), but also the presentation of a real obstacle or barrier to other microorganisms by competing directly for the host-cell-binding sites (as shown by Reid et al., 2001). Clearing a space to colonize by eliminating prior residents can be accomplished by production of antimicrobials or by production of molecules that facilitate the competitor's dispersal without actually killing them (see Modulation of Bacterial Settlement by Epibiotic Bacteria and Quorum Sensing and its Modulation below, Hibbing et al., 2010).

Each specific biofilm by its physical structure, its functional components, and their metabolic activity will affect host interactions differently. In the following we will concentrate on the aspects (i) how biofilms by physical insulation and metabolic filtration affect the host's access to matter and energy and (ii) how biofilms – mainly due to released infochemicals – modulate the interactions between host and further colonizers, potential consumers, and – very summarily – pathogens.

MODULATION OF THE ACCESS OF THE HOST TO RESOURCES (NUTRIENTS, GASES, LIGHT, INFOCHEMICALS, TOXINS)

Epibiotic biofilms constitute a physical and physiological barrier between their host and the environment. How biofilms at different stages of their development interfere with their substrate's surface properties in general and transfer of matter and energy through the fouled surface in particular has been investigated at great length for technical surfaces, such as reverse osmosis membranes in desalination plants, submerged optical, and other sensors or heat exchange devices (e.g., Winters and Isquith, 1979; Flemming, 1997; Baker and Dudley, 1998; Kerr and Cowling, 1998). Presumably, the passage of chemicals and radiation across these membranes is modulated by microfouling quite analogously to what is happening at the living surfaces of marine organisms covered by epibiotic biofilms. However, this insulating or filtering function of biofilms is much less studied in epibiotic associations because typically these biofilms cannot be maintained structurally and functionally intact in the absence of the host. Based on the few studies available (references in Wahl, 2008a) and extrapolated from the more technical studies mentioned before the following effects of epibiotic bacterial biofilms on their hosts have been shown or are plausible.

Physically, the biofilms represent the new functional interface between the host and environment replacing many properties of the host's surface, such as color, microtexture, or wettability by the corresponding biofilm properties (e.g., Becker and Wahl, 1991; Bers et al., 2006). Irradiation of optical sensors (eyes or more primitive photoreceptors) and of photosynthetic organelles (chloroplasts) may be reduced by the presence of biofilms (e.g., Philip-Chandy et al., 2000; Head et al., 2004). Bacterial biofilms only few weeks old may reduce the incoming light by over 50% (Wahl et al., 2010), which undoubtedly would severely affect the photosynthetic performance of primary producers and, consequently, their depth distribution. This investigation also highlighted that warming accelerates the formation of shading biofilms, putting a greater challenge on protective measures by the host. Without antimicrofouling defenses, at 25°C naturally establishing biofilms absorb 95% of the incoming light, virtually blinding fouled photoreceptors. It is likely, but unproven, that the diffusion of gases (CO_2, O_2) through the host's epidermis is compromised by biofilms. Thus, the metabolism of primarily heterotrophic biofilms will deplete O_2 and enrich CO_2 before they reach the host surface. Similarly, the access of the host to nutrients in the water column (nitrate, phosphate, bicarbonate, micronutrients, vitamins, amino acids, polycarbonates, etc.) can be hindered by reduced diffusion through or pre-emption by a biofilm (as suggested by

numerous studies on the role of biofilm in water purification, e.g., Terada et al., 2006). In contrast, some nutrients including vitamins or growth factors are provided to the host by epibiotic biofilms (e.g., Chisholm et al., 1996; Seyedsayamdost et al., 2011). In certain extreme environments (as cold seeps and black smokers) biofilms may constitute the trophic interface enabling the host to live on otherwise toxic compounds (e.g., Goffredi, 2010). Epibiotic microorganisms may interfere with the reception or release of infochemicals which serve communication between conspecifics or between interacting species (see below Modulation of Eukaryote Settlement by Epibiotic Bacteria). All these insulating (or degrading) effects of the biofilm can be beneficial when the factors warded off are potentially harmful to the host such as UVR, toxins or infochemicals used by searching foes (consumers, parasites, pathogens; e.g., Steinberg et al., 2011). Also, some larvae use the chemical cues emitted by characteristic biofilms on adult conspecifics for gregarious settlement (De Gregoris et al., 2012).

FOULING MODULATION BY EPIBIOTIC BIOFILMS

Surface modification

The characteristics of the substratum have a significant effect on the rate and extent of attachment of microorganisms (Donlan, 2001). Surface roughness and microtopographical features have been postulated as one aspect of mechanical antifouling defense mechanisms of some invertebrates, for example, by the development of small spicule-like or ripple-shaped structures (Bers and Wahl, 2004). Wettability of surfaces also affects, and in certain ranges hinders, attachment (e.g., Becker et al., 2000). This notwithstanding, colonizing organisms, and in particular bacteria, have evolved many mechanisms that allow them to colonize a host surface (Reid et al., 2001) and to form a biofilm on it. Those biofilms confer special properties to the surface of the substratum that may completely mask the properties, including the physical fouling-reducing surface properties just mentioned, of the underlying substratum itself (Donlan, 2001; Bers et al., 2006). Biofilm surfaces vary from smooth and confluent to rough and uneven with tall cell clusters interweaved by fluid-filled channels (see Nadell et al., 2009). Surface modifications by epibiotic biofilms comprise the alteration of the surface chemical composition and morphology, surface topography and roughness, the hydrophilic/hydrophobic balance, as well as the surface energy and polarity (Vladkova, 2009). The progressive recruitment of micro-colonizers and their production of a mucus extracellular polysaccharide matrix gradually covers the host's features which may facilitate settlement of macro-colonizers that previously discriminated against host's features (Costerton et al., 1995; Bers and Wahl, 2004; Vladkova, 2009). Mature biofilms will to some degree control the flux of energy and matter through the host's body surface (e.g., Costerton et al., 1987; Dobretsov et al., 2006b; Wahl, 2008b), altering the chemical properties of the boundary layer.

Biofilms comprise not only cells but also a myriad of compounds that these cells release into the biofilm matrix and the boundary layer (Nadell et al., 2009). By overlaying host attributes by its own chemical information a biofilm may promote further colonization by some or deter colonization by other potential foulers (see Joint et al., 2000; Dobretsov, 2009).

Modulation of bacterial settlement by epibiotic bacteria

Antagonism plays a significant role in shaping bacterial communities (Mangano et al., 2009). The production by microbes of secondary metabolites against potential competitors, predators, or antagonists may indirectly affect the host and its biological interactions with foulers and pathogens (e.g., Gil-Turnes et al., 1989; Armstrong et al., 2001; Steinberg et al., 2011). Space and nutrient limitation are enforcing surface dwelling microorganisms to evolve particular adaptive responses to prevent colonization or growth of potential competitors (Egan et al., 2008). From an ecological point of view, inhibitory interactions among bacteria inhabiting the same niche represent an interesting evolutionary strategy, conferring a selective advantage in competition, and acting as an effective control of microbial populations (Hentschel et al., 2001). In some cases, one organism may inhibit growth or metabolism of other organisms directly by excretion of a specific inhibitor (Wannamaker, 1980). In other cases the effect is indirectly or at least non-specifically mediated by the physiological activities of the organism producing, e.g., acids from the fermentation of sugars (Madigan and Martinko, 2006). Such responses may include induction of negative chemotaxis in potentially competing bacteria or the mentioned interference with processes leading to the irreversible attachment of cells to substratum (Boyd et al., 1999). The specific mediators playing a role in a bacterial antagonism range from rather complex substances (such as bacteriocins and enzymes) to simple molecules (such as ammonia, lactic acid, free fatty acids, and hydrogen peroxide; Wannamaker, 1980).

Bacteria producing antimicrobial and other bioactive compounds have been isolated from a range of marine invertebrates and algae including ascidians, bryozoans, corals, crustaceans, mollusks, sponges, tubeworms, etc. (**Table 2**). They have yielded a large number of new natural products as arenimycin, bacillistatins, bogorol, harman, lutoside, salinamides, sesbanimides, among many others (Acebal et al., 1998; Bultel-Poncé et al., 1998; Moore et al., 1999; Barsby et al., 2002; Aassila et al., 2003; Pettit et al., 2009; Asolkar et al., 2010). It should be cautioned at this point, that in the majority of studies "bioactivities" are not assessed at natural *in situ* concentrations of the compounds. One reason is that in many cases the research motivation was pharmacological rather than ecological, another reason being that metabolite concentrations in the boundary layer are difficult to determine. In these cases, an extrapolation of the *in vitro* results to a real function within the biofilm *in vivo* is problematic (e.g., Clare, 1996). The production of antimicrobial compounds is not restricted to a certain bacterial group but instead appears to be wide spread across various bacterial phyla (Penesyan et al., 2009), and is neither limited to a geographical region or habitat (see **Table 2**). Additionally, a single microorganism has the potential to produce many different compounds under different conditions (Bode et al., 2002). It is thought that a generalist bacterial species occupying a broad spectrum of environments (i.e., *Bacillus*, *Pseudoalteromonas*, or *Streptomyces*) would be more likely to benefit from producing broad spectrum antimicrobials or a cocktail of toxins targeting different potential competitors, while those organisms highly specialized for a given habitat (i.e., obligate epiphytes) may produce antimicrobials with narrower range, targeting specific competitors (Hibbing et al., 2010). Although production of bioactive

Table 2 | Antimicrobial activity of epibiotic bacterial strains isolated from different hosts.

Host	Total strains	Active strains	% of active strains	Test	Country	Reference
MACROALGAE						
Saccharina latissima	210	103	50	lb	Germany	Wiese et al. (2009)
Invertebrates 4spp., alga	400	140	35	lb, env	Scotland	Burgess et al. (1999)
Macroalgae 5 spp.	224	38	16.9	lb	Spain	Lemos et al. (1985)
Macroalgae 7 spp.	280	60	21	env	Scotland	Boyd et al. (1999)
Brown algae 9 spp.	116	23	20	lb, env	Japan	Kanagasabhapathy et al. (2006)
Red algae 9 spp.	92	31	33	lb, env	Japan	Kanagasabhapathy et al. (2008)
Macroalgae 2 spp.	325	39	12	lb	Australia	Penesyan et al. (2009)
Ulva lactuca	10	6	60	env	Fiji	Kumar et al. (2011)
INVERTEBRATE						
Acropora formosa	354	36	10	lb	India	Chellaram et al. (2011)
Anoxycalyx joubini	38	–	90	env	Antarctica	Mangano et al. (2009)
Balanus amphitrite	28	4	14.3	lb	India	Jebasingh and Murugan (2011)
Bryozoa 14 spp.	340	101	29.7	lb	MS, BS	Heindl et al. (2010)
Coral 2 spp.	352	46	13	lb, env	India	Gnanambal et al. (2005)
Coral 9 spp.	78	19	24.3	lb, env	Israel	Shnit-Orland and Kushmaro (2009)
Echinoderms 2 spp.	9	9	100	lb	India	
Favia palida	335	41	13	lb	India	Chellaram et al. (2011)
Haliclona simulans	52	30	57.6	lb	Ireland	Kennedy et al. (2009)
Haliclona sp.	56	8	14.3	lb	Indonesia	Radjasa et al. (2007)
Invertebrates 14 spp.	105	14	13	lb, env	Australia	Wilson et al. (2011)
Invertebrates spp.	290	54	18.6	lb	Venezuela	Castillo et al. (2001)
Lissodendoryx nobilis	37	–	62.2	env	Antarctica	Mangano et al. (2009)
Mycale adhaerens	20	15	75	env	Hong Kong	Lee and Qian (2004)
Penaeus monodon	185	49	26.3	env	India	Shakila et al. (2006)
Petrosia ficiformis	57	5	8.7	lb, env	Italy	Chelossi et al. (2004)
Sarcophyton sp.	98	6	6.3	env	Indonesia	Sabdono and Radjasa (2006)
Sponge 2 spp.	238	27	11.3	lb	MS	Hentschel et al. (2001)
Sponge 4 spp.	28	4	14.3	lb	India	Nair et al. (2011)
Sponge 11 spp.	20	10	50	lb	MS	Abdelmohsen et al. (2010)
Sponge 9 spp.	158	12	7.6	lb	Brazil	Santos et al. (2010)
Sponge 10 spp.	2562	283	15.2	lb	MS	Muscholl-Silberhorn et al. (2008)
Sponge 4 spp.	75	16	21	lb	India	Anand et al. (2006)
Sponge 5 spp.	26	21	80.7	lb	India	Gandhimathi et al. (2008)
Sponge 4 spp.	94	58	61.7	lb	India	Dharmaraj and Sumantha (2009)

Where lb, laboratory test strains, env, wild strains, BS, Baltic Sea, MS, Mediterranean Sea. Note that only exceptionally natural concentrations were known, and test concentrations may differ substantially from these.

compounds is a characteristic feature of some bacteria and may largely promote the colonization of and competition on host surfaces (Holmström and Kjelleberg, 1999; Patel et al., 2003; Rao et al., 2005), they can be costly in terms of resource allocation, diverting energy away from growth and reproduction (Kumar et al., 2011). To effectively inhibit competitors, the antibiotic must be produced in sufficient quantity, and this may require the concerted effort of a population. Accordingly, antibiotic production often is regulated by a QS mechanism (Hibbing et al., 2010). Antimicrobials have been postulated to be (in nature) rather signaling molecules within species than chemical weapons (Hibbing et al., 2010). Supporting this view, antibiotics are often produced at sub-inhibitory concentrations, the metabolic costs of their production are relatively high, and many bacteria have a capacity for fast evolutionary development of tolerance against antimicrobials (Hibbing et al., 2010). The presumed predominance of an informational function of secondary metabolites has led to the emergence of a new field of research named neuroecology (e.g., Steinberg et al., 2011). Given the ability of bacteria to escape potentially harmful environments in response to sub-lethal concentrations of such chemoeffectors, the metabolites responsible for mediating antifouling mechanisms may well be overlooked using standard antimicrobial assays (Young and Mitchell, 1973). The abundance of behavioral (deterrent) effects relative to lethal (antibiotic) effects in defensive metabolites has been shown for marine invertebrates and algae (Wahl et al., 1994; Engel et al., 2002). With conventional testing these ecologically important activities would go undetected.

Bacteria producing antibiotic substances are more prevalent in epibiotic biofilms than in other habitats, such as seawater (Mearns-Spragg et al., 1998; Zheng et al., 2005; Kanagasabhapathy et al.,

2008, see **Table 2**). Furthermore, some bacteria may actually produce active compounds only when they are cultivated on surfaces, as for example one strain of *Bacillus licheniformis*, which lost the bioactivity when it was cultured in a liquid medium instead of on agar (Yan and Boyd, 2002; Matz et al., 2008). It cannot be decided now whether this is a result of a better energy supply in the epibiotic microhabitat or of enhanced competition in the biofilm (Kanagasabhapathy et al., 2008; Mangano et al., 2009; Steinberg et al., 2011).

There is still a long way to go if we wish to understand what kind of compounds bacteria produce under which set of environmental parameters *in situ*. Their behavior may be affected by many factors (Bode et al., 2002) including chemical signal from the host (e.g., Dworjanyn and Wright, 2006; Steinberg et al., 2011), and it is difficult to know whether, when, or how micro-epibionts possibly protect their hosts. Bacteria frequently change their metabolic profile once they are outside of their natural habitat because of altered growth conditions and lack of selective pressure (Mangano et al., 2009). For example, a marine actinomycete (strain SS-228) was shown to produce an antibiotic compound only when the growth medium was supplemented with *Laminaria* sp., a macroalga common in the habitat from which the strain was obtained (Okazaki et al., 1975).

Also, strain specific variation of the production of antibiotics is a well documented phenomenon, e.g., in *Pseudoalteromonas* and *Bacillus* strains (see Todorova and Kozhuharova, 2010; Vynne et al., 2011) and this may have two reasons: First, bacteria quickly adapt to the environment and the production of secondary metabolites relies on many different factors (Bode et al., 2002). Second, plasmids, transposons, or phages may enable the mobilization and transfer of biosynthetic operons between different bacterial strains and even across the species barrier (Martin and Liras, 1989), which in combination with rapid growth rates and large population sizes results in the introduction of many unique mutations that even at low frequencies may rise to variants that are more adapted or biologically active (Nadell et al., 2009; Hibbing et al., 2010).

Modulation of eukaryote settlement by epibiotic bacteria

Biofilms may either enhance or inhibit settlement of propagules (reviewed by Dobretsov et al., 2006b; Qian et al., 2006; Prendergast et al., 2009; Hadfield, 2011, **Table 3**). Since the response to a biofilm differs among potential settlers, the influence of a biofilm on recruitment also has a selective aspect. All components of marine biofilms (bacteria, diatoms, fungi, and protozoa) may potentially affect larval and algal settlement through physical modification of surfaces and production and release of molecular cues or deterrents.

Several independent studies have shown that strains of epibiotic bacteria associated with sponges (Lee and Qian, 2003), soft corals (Dobretsov and Qian, 2004), tunicates (Szewzyk et al., 1991; Egan et al., 2008), and algae (Dobretsov and Qian, 2002) can be grouped into three functional groups based on their bioactivity toward a given macrofouler. They can be inductive (induce settlement), non-inductive or neutral (do not induce settlement), and inhibitive (significantly reduce larval settlement).

Almost all (epibiotic) biofilms in the sea are multispecies (Wieczorek and Todd, 1998; Dobretsov, 2010). Their effects may differ from those of monospecific bacterial biofilms (Tran and Hadfield, 2011), which possibly explains the fact that most studies of natural assemblages so far only reported inhibitory effects (references within Dobretsov et al., 2006b and this review). For example, artificial biofilms composed of 11 "inductive," "neutral," and "inhibitive" strains from the soft coral *Dendronephthya* sp. at a 1:1:1 ratio inhibited larval settlement of *Hydroides elegans* and *B. neritina* in a laboratory experiment (Dobretsov and Qian, 2004, **Table 3**). The bioactivity of multispecies biofilms depends not only on the presence of particular bacterial taxa but also on their proportional abundance (Lau and Qian, 1997; Harder et al., 2002; Dahms et al., 2004). Further, bacteria potentially produce different types or quantities of settlement modulating compounds under laboratory conditions and in the field, as the biotic and abiotic environment usually determines bacterial behavior (reviewed by Dobretsov et al., 2006b). Below we will provide some examples of epibiotic bacteria that induce or inhibit the settlement of potential eukaryote foulers through a release of bioactive compounds. It should be noted at this point, that this infochemical (or "neuroecological") interaction is bidirectional: numerous propagules cue on biofilm signals to detect suitable habitats or reject unsuitable ones (e.g., Hadfield, 2011) and it is difficult to differentiate this behavior from a "repellent" or "attractant" activity of a bacterial metabolite. In most cases, these aspects are probably the two sides of the same coin.

Numerous examples demonstrate that epibiotic biofilms induce larval settlement of cnidarian, mollusks, and polychaete species (reviewed by Wieczorek and Todd, 1998; Prendergast et al., 2008). In a laboratory experiment, biofilms from the green filamentous alga *Cladophora rupestris* attracted larvae of *Mytilus edulis*, while biofilms from the brown alga *Laminaria saccharina* repelled them (Dobretsov, 1999). *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 isolated from the surfaces of marine seaweeds and mussels significantly increased larval settlement of *Perna canaliculus* (Ganesan et al., 2010). Bacteria belonging to the genera *Vibrio* and *Pseudoalteromonas* associated with the shells of *B. amphitrite* induced gregarious settlement of the host species (De Gregoris et al., 2012). In this study, even small variations in the proportion of the species of the biofilms produced different effects on larval settlement. While the phenomenon of settlement induction driven by epibiotic bacteria is widespread (Dobretsov, 2009), to date only few inductive compounds from epibiotic bacteria have been isolated. This includes tetrabromopyrrole that induced larval attachment and metamorphosis of the acroporid coral larvae *Acropora millepora*, and was produced by *Pseudoalteromonas* strains associated with the crustose coralline algae (Tebben and Tapiolas, 2011).

It has been previously proposed that mainly *Pseudoalteromonas* species inhibit settlement of propagules (Holmström and Kjelleberg, 1999; Holmström et al., 2002). Nowadays, we know that there is no correlation between the inhibition of larval settlement and bacterial phylogeny (reviewed by Dobretsov et al., 2006b, **Table 3**). In a pioneer work on bacteria associated with the shells of the barnacle *B. amphitrite*, Mary et al. (1993) demonstrated that 12 out of 16 isolates inhibited larval settlement. In another study, the activity of 10 *Pseudoalteromonas* species isolated from marine sponges, algae, and tunicates on the settlement of larvae of the

Table 3 | Inhibition of larval settlement by epibiotic bacteria and compounds from these, ? – no data available.

Host	Bacteria	Effective against	Active compound	Reference
Barnacle *B. amphitrite*	*Vibrio*, *Alteromonas*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas*	Barnacle *B. amphitrite*	?	Mary et al. (1993)
Algae, sponges, and ascidian *Ciona intestinalis*	*Pseudomonas aurantia*, *P. citrea*, *P. piscicida*, *P. rubra*, *P. undina*, *P. ulvae*, *P. haloplanktis*, *P. luteoviolacea*, *P. tunicata*, *P. nigrifaciens*	*Polychaeta, Hydroides elegans*, barnacle *Balanus amphitrite*, and ascidian *Cliona intestinalis*	?	Holmström and Kjelleberg (1999), Holmström et al. (2002)
Nudibranch *Archidoris pseudoargus*	*Pseudomonas* sp.	Barnacle *Balanus amphitrite*	Phenazine-1-carboxylic acid, 2-n-hyptyl quinol-4-one, 1-hydroxyphenazine phenazine-1-carboxylic acid, pyolipic acid	Burgess et al. (2003)
Green alga *Ulva reticulata*	*Vibrio alginolyticus*	*Polychaeta, Hydroides elegans*	Polysaccharide >200 kDa consist of glucose, mannose, galactose, and glucosamine	Dobretsov and Qian (2002), Harder et al. (2004)
Soft coral *Dendronephthya* sp.	*Vibrio* sp., uncultured *Ruegeria*, unidentified *a-Proteobacterium*	*Polychaeta, Hydroides elegans*, bryozoan *Bugula neritina*	?	Dobretsov and Qian (2004)
Sponge *Halichondria okadai*	*Alteromonas* sp.	Barnacle *B. amphitrite*	Ubiquinone	Kon-ya et al. (1995)
Ascidian *Stomozoa murrayi*	*Acinetobacter* sp.	Barnacle *B. amphitrite*	6-bromindole- 3-carbaldehyde	Olguin-Uribe et al. (1997)
Green alga *Ulva australis*	*Pseudoalteromonas tunicata* and *Phaeobacter* sp. strain 2.10	Bryozoan *Bugula neritina*	?	Rao et al. (2007)
Brown alga *Fucus serratus*, *F. vesiculosus*, and the red alga *Polysiphonia stricta*	Natural communities, *Photobacterium halotolerans* and *Ulvibacter litoralis*, *Shewanella basaltis*, *Pseudoalteromonas arctica*, *Shewanella baltica*, and *Bacillus foraminis*	Barnacle *Amphibalanus improvisus*	?	Nasrolahi et al. (2012)
Sponge *Lissodendoryx isodictyalis*	*Winogradskyella poriferorum*	Polychaete *H. elegans* and barnacle *B. amphitrite*	Poly-ether AE	Dash et al. (2009), Dash et al. (2011)

barnacle *B. amphitrite* and the polychaete *H. elegans* has been investigated in laboratory experiments (Holmström et al., 2002). Only *P. tunicata*, *P. citrea*, and *P. ulvae* inhibited settlement of both larval species and the bacterium *P. tunicata* was the best performing bacterium. Surprisingly, *P. tunicata* isolated from Baltic macroalgae did not inhibit settlement of *Amphibalanus improvisus* larvae (Nasrolahi et al., 2012). In contrast, monospecific bacterial films of *Shewanella baltica* and *Pseudoalteromonas arctica* associated with the red alga *Polysiphonia stricta*, *Photobacterium halotolerans*, and *Ulvibacter litoralis* isolated from *F. serratus*, and *S. baltica* and *Bacillus foraminis* isolated from *F. vesiculosus* reduced the attachment of cyprids of *A. improvisus* (Nasrolahi et al., 2012). The strongest inhibitory effect was obtained with isolates from *P. stricta*. Biofilms and conditioned seawater from seven isolates obtained from the alga *U. reticulata* reduced settlement of *H. elegans* larvae (Dobretsov and Qian, 2002) and the antifouling compound from the epibiotic *Vibrio* sp.2 (identified later as *V. alginolyticus*) was identified as a large >200 kDa polysaccharide consisting of glucose, mannose, galactose, and glucosamine (Harder et al., 2004). In another study, *Vibrio* sp., unidentified *Ruegeria* and *a-Proteobacterium* isolated from the soft coral *Dendronephthya* sp. inhibited larval settlement of *H. elegans* and *B. neritina* (Dobretsov and Qian, 2004). Natural biofilms isolated from their host, the brown alga *F. vesiculosus*, with their original composition intact, inhibited the settlement by barnacles – a protective activity which tends to be jeopardized at stressfully high and low temperature by structural shifts in the biofilm (Nasrolahi et al., 2012).

Only few antifouling compounds originating from epibiotic bacteria have been isolated and identified (Dobretsov et al., 2006b; **Table 3**). One of the first antifouling compounds identified as ubiquinone was isolated from *Alteromonas* sp., a marine bacterium associated with the sponge *Halichondria okadai* (Kon-ya et al., 1995). *Acinetobacter* sp., isolated from the surface

of the ascidian *Stomozoa murrayi*, produces 6-bromindole-3-carbaldehyde that inhibits settlement of cyprid's larvae in the barnacle *B. amphitrite* at concentrations of 10 mg ml^{-1} (Olguin-Uribe et al., 1997). Phenazine-1-carboxylic acid, 2-*n*-hyptyl quinol-4-one, 1-hydroxyphenazine-1-carboxylic acid, and pyolipic acid produced by the epibiotic bacterium *Pseudomonas* sp. associated with the nudibranch *Archidoris pseudoargus* inhibited *B. amphitrite* settlement (Burgess et al., 2003). Six poly-ethers A–E were isolated from *Winogradskyella poriferorum* isolated from the Bahamian sponge *Lissodendoryx isodictyalis* (Dash et al., 2009, 2011). These compounds inhibited settlement of the barnacle *B. amphitrite* and the bryozoan *B. neritina* but did not produce any adverse effects on the phenotypes of zebra fish embryos, which makes them promising candidates for antifouling applications.

Are epibiotic bacteria and their compounds able to protect their host in the natural environment? Given that "bioactivity" is concentration dependent, as we mentioned before, it is extremely difficult to simulate *in vitro* the mostly unknown *in situ* concentrations of bacterial metabolites in or on the biofilms and a clear answer is therefore not possible in studies where bacterial extracts were tested. When biofilms are tested *in vivo*, preferably in multispecies composition similar to the natural epibiotic biofilm (as e.g., in Nasrolahi et al., 2012), the answer is more straightforward. The bacteria *P. tunicata* and *Phaeobacter* sp. strain 2.10 (formerly *Roseobacter gallaeciensis*) associated with the alga *U. australis* can inhibit larval settlement at densities of 10^3 to 10^5 cells cm^{-2}, which is similar to the densities of these bacteria under the natural conditions (Rao et al., 2007). Thus, at least in the cases of the marine macroalgae *F. vesiculosus* and *U. australis* the epibiotic biofilms seem to contribute to the host's defense against macrofouling. It is likely that these cases are not exceptional.

Quorum sensing and its modulation

Quorum sensing is a cell-cell communication mechanism that allows bacteria to coordinate settlement, swarming, reproduction, biofilm formation, stress resistance, dispersal, and production of secondary metabolites (Waters and Bassler, 2005; Irie and Parsek, 2008; Steinberg et al., 2011). During this process, bacteria produce, release, and perceive small chemical signals named autoinducers. When the concentration of these signals in the environment reaches the threshold level, this triggers expression of target genes and change in the behavior of bacteria. There are various QS signaling systems used by Gram negative and Gram-positive bacteria, but the best known and characterized one is based on the production and perception of *N*-acyl homoserine lactones (AHLs) in Gram negative bacteria. Some Gram negative bacteria, like *Pseudomonas* spp. and *Vibrio* spp. produce multiple QS-signals (reviewed by Paul and Ritson-Williams, 2008; Dobretsov et al., 2009).

Most studies of settlement induction by biofilms have been realized on non-living surfaces. Their results cannot always be extended to host-epibiont interactions, as epilithic microbial communities usually differ in composition (and metabolomic activity) from epibiotic assemblages. However, if an identified settlement cue is released by epiphytic or epizooic microorganisms, an effect on settlement may at least be expected. This is the case with AHLs. Various bioactive AHL's are generated by approximately 30% of the bacteria associated with corals (Golberg et al., 2011), as well as by microorganisms associated with sponges (Taylor et al., 2004) and seaweeds (Berger et al., 2011). Some bacteria, like *Bacillus* spp., can produce enzymes such as AHL-acylase and AHL-lactonase that hydrolyze AHL signals and make QS impossible (reviewed by Dobretsov et al., 2009). These enzymes can be used by epibiotic bacteria in order to outcompete other bacterial species.

The behavior of various seaweed-associated bacteria is affected by AHL and by inhibitors of AHL-mediated QS (Maximilien et al., 1998), which also confirms that marine epibiotic communities produce and use AHL signals. AHL signals generated by artificial (Joint et al., 2002) and natural (Tait et al., 2009) biofilms attract zoospores of the green macroalga *Ulva intestinalis*, a facultative epiphyte on various seaweeds and eelgrass. There is also evidence that bacterially produced AHL's modulate the interaction of the red alga *Gracilaria chilensis* and its red algal epiphyte *Acrochaetium* sp. by controlling spore release in the latter (Weinberger et al., 2007). Similarly, out of 96 bacterial strains isolated from the brown alga *Colpomenia sinuosa* 12% inhibited AHL-mediated QS (Kanagasabhapathy et al., 2009) that induces spore release and spore settlement of certain algal epiphytes. The role of QS in controlling infections is just emerging (Campbell et al., 2011).

Given the important role of QS in bacterial and bacteria-alga signaling it is not surprising that some basibionts have learned to suppress this communication in order to control epibiosis and infections (reviewed by Dobretsov et al., 2009; Goecke et al., 2010; Steinberg et al., 2011).

FEEDING MODULATION

The modulation (enhancement, reduction) of feeding by macroepibionts is well investigated (e.g., Wahl, 2008a). In contrast, to which extent epibiotic biofilms or other associated bacteria contribute to the regulation of feeding on their host is largely unknown. Many marine organisms possess secondary metabolites of various functions that are structurally similar to known microbial metabolites, but so far only relatively few studies have rigorously demonstrated microbial production of these metabolites (Piel, 2004, 2009). Although chemical defense against consumers is a common trait among seaweeds, sponges, bryozoans, tunicates, and other members of sessile, soft-bodied marine taxa (Paul and Ritson-Williams, 2008; Paul et al., 2011), the evidence for contribution from sym- or epibiotic bacteria to this defense is limited. An example of a defensive symbiosis was discovered on coral reefs in Papua New Guinea, where epibiotic microbial communities dominated by Cyanobacteria of the genus *Synechococcus* protect their host – isopods of the genus *Santia* – from fish predators (Lindquist et al., 2005). The isopods consume these photosymbionts that live on their surface and warrant their growth by staying in sunlit areas which should make them more vulnerable to fish predators, especially so because the epibionts are brightly pigmented. However, the epibionts produce chemical deterrents that strongly detract the predators.

Not only mutualistic, but also antagonistic microorganisms in some cases modulate the interactions between marine organisms and their consumers. For example, the activation of innate immune responses in *F. vesiculosus* through challenge with cell wall matrix degradation products resulted in a reduction of

palatability to the omnivorous isopod *Idotea baltica* (Kruse et al., 2009). Cell wall matrix degradation without simultaneous tissue destruction obviously results from pathogen attacks rather than predator attacks, and the innate immune system of brown seaweeds is known to fend off opportunistic cell wall macerating pathogens (Küpper et al., 2002). In brown seaweeds pathogen attacks may thus induce not only anti-pathogen, but also anti-herbivore defenses. The scarcity of reports on effects of epibiotic bacteria on the consumers of hosts should, however, not be interpreted as a scarcity of such effects.

BIOFILM-DRIVEN MODULATION OF INFECTIONS

Various diseases of marine organisms are caused by opportunistic pathogens. For example, Vibrionaceae are well known as opportunistic pathogens in algae (Weinberger et al., 1994; Largo et al., 1995), crustaceans (Selvin and Lipton, 2003), mollusks (Liu et al., 2001; Paul-Pont et al., 2010), and fish (Martinez Diaz and Anguas Velez, 2002; Tian et al., 2008; Ye et al., 2008; Zhao et al., 2010). Under most circumstances, these Vibrionaceae are harmless to their hosts, but under specific – stressful – conditions they may turn virulent. Antibiotically active marine epibionts have successfully been tested as control agents of opportunistic pathogens in fish aquaculture (Planas et al., 2006). Correlative studies have repeatedly suggested that a structural shift in associated bacteria co-occurs with an infection of the host (e.g., Frias-Lopez et al., 2002; Pantos et al., 2003). Only very recently a more mechanistic approach to the host – epibacteria – pathogen interactions in a red macroalga was undertaken showing that warming stress led to a reduction in QS-suppressing furanones resulting in enhanced infections and bleaching of the host (Steinberg et al., 2011). Nonetheless, outside the few mentioned studies, relatively little hard evidence of symbiotic microbes defending their host against microbial infection in non-artificial systems has arisen since early ground breaking studies (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992), who demonstrated that bacterial epibionts protect crustacean eggs from infection by an oomycetic pathogen. This may perhaps simply be due to a pertaining lack of studies. However, a clear cut distinction of environmental impact upon the three cohorts of mutualistic, antagonistic, and commensalistic microorganisms that are associated with a host is extremely difficult under most conditions, which may also explain the rarity of published data.

ENVIRONMENTAL FACTORS AFFECTING MICROBIAL EPIBIOSIS

Biofilms functionally represent a new "skin" to the host organism and we have shown in the foregoing discussion that this skin has some potential to modulate the host's abiotic and biotic interactions. The composition of epibiotic biofilms may be host-specific to some degree (e.g., Lachnit et al., 2010; Goecke, 2011). Any compositional change in the biofilm may, but not necessarily does (Burke et al., 2011b), affect biofilm functions and, ultimately, the ecology of the host. Environmental changes (seasonality, disturbances, stress gradients, climate change) may affect biofilm composition directly or via physiological responses of the host which, in turn, lead to changes of the conditions in the boundary layer microhabitat.

Stress in its widest meaning, i.e., any combination of environmental variables reducing a species' performance (Wahl et al., 2011), is omnipresent, at least with regard to single variables. Rarely are all requirements of an organism – temperature, light, salinity, pH, nutrients, etc. – at their optimum value. Species are adapted to tolerate sporadic or rhythmic deviation from optimum settings which are typical for their habitat (e.g., Sanford and Kelly, 2010). However, this tolerance often goes along with a decrease in performance. If performance shifts are unequal among interaction species, these interactions will shift constituting an ecological lever which buffers or enhances the impact of environmental stress (Wahl, 2008b; Fabricius et al., 2011; Monaco and Helmuth, 2011). Environmental stress will affect the interaction between host and biofilm, but also among the components of the latter (Harder et al., 2012).

For non-epibiotic biofilms it has been shown repeatedly that they may vary among habitats and season even at small spatial scales (e.g., Thompson et al., 2005; Hung et al., 2007; Anderson-Glenna et al., 2008). The abundance and composition of a biofilm on a given substratum is the combined result of the regional composition of the pool of potential colonizers, selective recruitment onto a surface, the activity of consumers, substrate characteristics, and abiotic factors (temperature, salinity, nutrients, irradiation, pH, etc.) which determine the presence and performance of single strains and the interactions among the strains (e.g., Railkin, 2004). Composition and density of the pool vary seasonally and with an exchange of water body (e.g., upwelling). Selective recruitment onto a living surface will depend on the surface's properties (e.g., nutrients, defenses). Succession of the epibiotic biofilm community is driven by continued recruitment and interaction among biofilm components which in turn are determined by conditions in the boundary layer and activities of the host.

While the environmental control on bacterioplankton and on non-epibiotic biofilms is well studied, we know substantially less on how environmentally driven changes in the host's performance affect epibiotic biofilm composition and their role in the host's ecology. There is some evidence that under warming stress the prevalence of diseases increases (e.g., Ainsworth and Hoegh-Guldberg, 2009; Mydlarz and McGinty, 2010; Campbell et al., 2011). Either pathogens become more abundant, or biofilm components turn virulent, or the defensive capacities of biofilm and/or host are weakened. Grimes (2002) and Vezzulli et al. (2012) presented data that hint at increasing abundance and virulence of bacterial opportunists belonging to the Vibrionaceae in coastal waters during the last decades and suggested a link with global warming. Numerous bacterial strains that are associated with marine organisms and form biofilms on their surfaces are known to produce antibiotics (see section "Modulation of bacterial settlement by epibiotic bacteria") and there is increasing evidence that these microorganisms contribute significantly to the host's resistance against macro- and micro-foulers (see section "Modulation of eukaryote settlement by epibiotic bacteria"). In this light a relevant contribution of the same bacteria to the host's resistance against opportunistic or even obligate microbial pathogens appears as possible.

Interactive effects of opportunistic pathogens, mutualistic microorganisms, and abiotic stress have been observed in corals

(Ben-Haim, 2003; Bourne et al., 2007; Sussman et al., 2008; Sunagawa et al., 2009), in which bleaching-related disease symptoms appear after shifts in the bacterial community composition toward a strongly increased abundance of Vibrionaceae and Alteromonadaceae (Bourne et al., 2007). Under non-stress conditions these potential pathogens are suppressed by antibiotics secreted by beneficial bacteria in the coral mucus (Ritchie, 2006). Under temperature stress the density of beneficial bacteria decreases, which is correlated with a loss of the protective properties of the mucus. Apparently warming triggers a primary loss of protective bacteria and/or a stimulation of overgrowth by non-protective and pathogenic commensals that are characterized by a strong proteolytic activity (Sussman et al., 2008). It is probably this shift toward pathogenic bacteria that prevents the recovery of corals from temperature stress.

Seasonal warming increases the density of bacteria, pathogenic or not, by a factor 10 in the Baltic Sea (0.45×10^6 per ml^{-1} in winter and 5.67×10^6 per ml in summer, Zimmermann, 1977). At the same time, the phylogenetic composition of this pool varies substantially among seasons (Lachnit et al., 2010; Koskinen et al., 2010). During a warming event (and presumably in the course of climate change) the density, phenology, activity, and composition of bacterioplankton (i.e., the pool of potential colonizers) change (e.g., Hoppe et al., 2008). As the level of activity as well as physiological status of potential host organisms is affected by environmental conditions, we can expect their surface properties (fouling modulating metabolites, exudates, O_2^-, and pH values) to vary with abiotic conditions. It is not surprising then that – driven by these two factors or more – the epibiotic bacterial community on a given host species may vary strongly with season (e.g., Lachnit et al., 2010). To some extent, the composition of biofilms among co-occurring conspecific hosts also differs (Lachnit et al., 2010; Burke et al., 2011b) which may or may not lead to altered functioning of the biofilms. Functional properties of and functional differences among epibiotic biofilms are severely understudied. We do not really know what an epibiotic microorganism "does," e.g., which compounds it anabolizes, or which of the compounds transiting between host and environment it catabolizes or transforms to new compounds. Environmental stress, i.e., a particularly strong deviation of one or more variables from an organism's optimum, may affect the biofilm bacteria directly (see above) or indirectly via shifts in the host's defenses or exudate quantity or quality. As a functional analogy, indirect stress effects on the intestinal biofilm of mammals with a multitude of possible health issues are well studied (e.g., Nettelbladt et al., 2003). Adverse environmental conditions such has heat waves or low light may affect the efficacy of chemical antimicrofouling defenses in the bladder wrack *F. vesiculosus* (Rohde et al., 2008; Wahl et al., 2010). Under experimental stress (warming, shading, desalination) the composition of the epibiotic biofilm on the bladder wrack re-organizes (S. Stratil, GEOMAR, pers. comm.). This may have direct consequences for *Fucus* if the bacteria involved in this change provide vitamins or nutrients, or deliver other goods to the host, an aspect unstudied for this host species, but established for the related *F. spiralis* (Fries, 1977, 1982, 1993). However, the indirect effects may be as large or larger. We have mentioned earlier (see "Fouling modulation by epibiotic biofilms") that some bacteria epibiotic on *Fucus* hinder further fouling by other bacteria or by barnacles (Nasrolahi et al., 2012). There are reports on epibiotic bacteria suppressing infections (Reid et al., 2001) or causing them (Wang et al., 2008; Fernandes et al., 2011). Although to be expected, a modulation by biofilms of consumption pressure on the host has not yet been investigated. These examples, and those given earlier in this article, show that a compositional shift in the epibiotic biofilm probably is ecologically not trivial. Depending whether beneficial or detrimental strains change in abundance in the biofilms, such a re-structuring under stress may buffer or enhance the more direct stress effects on the host. An even more direct response to environmental shifts than re-structuring of the biofilm could be metabolic shifts within a structurally stable biofilm with its components doing more, less, or different things than under another set of environmental variables (e.g., Steinberg et al., 2011).

Apart from the local stress regimes that native organisms are expected to be adapted to, a species may be subject to stress gradients in space and time. Stress gradients in space can be found along the distributional axis from core to margin of a host species' range, or when a species is translocated during a bioinvasion process. Stress gradients in time are associated with global change. As the coastal oceans are gradually shifting toward a warmer, nutrient richer, less oxygenated, sourer status, conditions in a given locality may turn more stressful. In addition, the introduction of alien species may represent novel interactions for the host. Bioinvasion research in the past has focused on macroorganisms. However, it was shown that invasive algae may carry along their associated biofilm (Meusnier et al., 2001) and that huge amounts of allochthonous microorganisms are continuously imported via ballast water and biofilms on moving artificial substrata such as ships (Drake et al., 2007) and, presumably but unexplored, drifting litter. Epibiotic bacteria may hitchhike over vast distances with their host (e.g., Grossart et al., 2010). Since the conditions in the boundary layer on the host's surface, the microhabitat of the epibiotic bacteria, is very much controlled by the host, attached bacteria can be seen as traveling in a space craft across potentially adverse outside conditions.

With increasing stress on the host and/or the epibiotic bacteria both the identity of the biofilm components may change, as well as their behavior, turning neutral or beneficial epibionts into pathogens for instance (e.g., Pruzzo et al., 2008; Feeding Modulation, but, see Burke et al., 2011a). Structural and/or functional changes in the biofilm may buffer or enhance environmental stress on the host.

CONCLUSION AND SUGGESTIONS

What do we know?

1. Biofilm bacteria and free-living bacteria are two states of aggregation of one regional pool of bacteria. Through attachment-detachment cycles there is intense exchange between the two compartments. In the biofilm state, interactions, metabolism, reproduction, and genetic exchange are substantially accelerated compared to the free-living state. Biofilms form at interphases, mostly solid/liquid but also liquid/gas or liquid/liquid (of different densities).

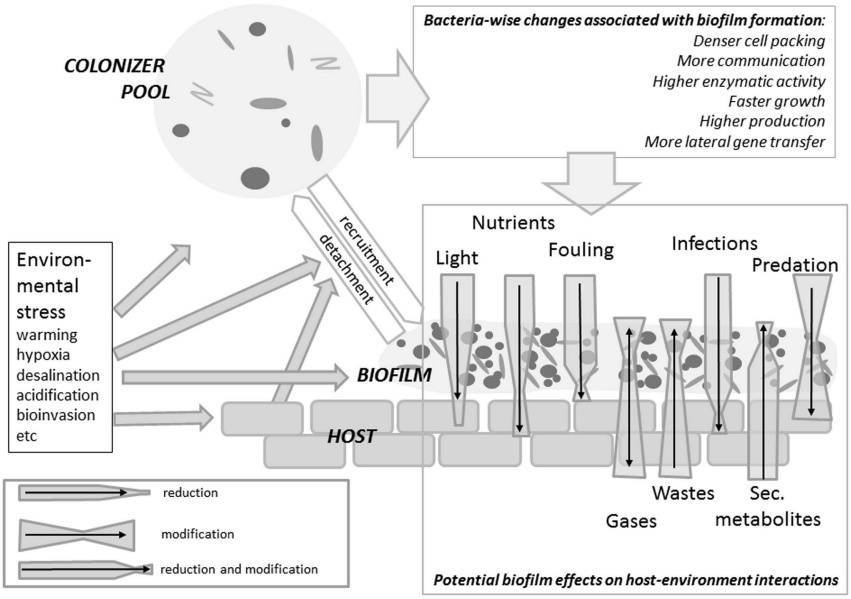

FIGURE 2 | Summary of biofilm impact on the host varying from detrimental to beneficial effects according to the epibiont's identity, the type of interaction considered and the environmental conditions. Via a recruitment/detachment equilibrium – controlled by environmental and host traits – epibiotic bacterial communities are connected to the free water phase. When forming a biofilm, bacteria experience a boost in activity and interactions. The host will experience a certain reduction in irradiation. Fouling, infections and predation will be affected by the presence of a biofilm, but extent and even sign of these effects are context-specific. An algal host will experience a reduction or an enhancement in nutrient availability depending on whether the autotrophic, respectively heterotrophic components prevail in the biofilm. Wastes and secondary metabolites (including infochemicals) may be metabilized by the biofilm.

2. All marine organisms bear epibiotic biofilms which range from sparse to dense and from monospecific to highly diverse.
3. These epibiotic biofilms have a huge potential to affect the biology, ecology, and fitness of their host. Many direct and indirect effects of epibiotic biofilms have been described, many more can be expected to exist (**Figure 2**).
4. Density and composition of epibiotic biofilms vary at different scales: among host species, among conspecific host individuals, among body regions of a host individual, among habitats, and among seasons. Structural differences among biofilms may or may not affect their function.

What we need to explore:

1. Metabolomics: newly emerging techniques such as DESI-MS coupled to MS-MS allow characterizing the surface chemical landscape of a biofilm, i.e., the compounds produced by the net metabolism of the epibacterial community (e.g., Prince and Pohnert, 2010; Nylund et al., 2011; Goulitquer et al., 2012). This chemical landscape should be characterized for the same biofilms under varying environmental conditions and host activities to asses the scope of metabolome fluctuations. Structure analysis of single compounds may allow searching for described functions.
2. "Soft" surface extraction techniques (Nylund et al., 2007; Lachnit et al., 2010; Saha et al., 2011) and non-intrusive analytical techniques (e.g., confocal resonance Rahman spectroscopy; Grosser et al., 2012) allow for the isolation and analysis of compounds in the boundary layer. Bioassays should be used to verify activities of whole or fractionated extracts against foulers, consumers, pathogens.
3. The combination of the first two approaches will shed light on the relationship between structure and function of epibacterial communities. This approach is more direct and more powerful than the metagenomics of functional genes (Burke et al., 2011b). It may help resolve the central question to which extent the observed structural differences in biofilms at numerous spatial and temporal scales are associated with functional differences. After all it is the function of the biofilm which matters for the host and the organisms interacting with it, not the identity of the biofilm components.
4. New techniques should be optimized to separate host and biofilm. This would permit to assess, at least for a short while, how the two components fare in the absence of the partner component. Any change in performance following the separation could deliver valuable hints at their interactions when united.
5. Once the ecological value of epibacterial communities is defined we may want to know to which degree and how a host can influence community services of its bacterial biofilm – via its composition and its activity. It is conceivable that a host chemically promotes fouling by beneficial strains, or by strains which in turn promote the establishment of a beneficial biofilm. Furthermore, the host could influence biofilm activities by exuding certain nutrients or infochemical (including QS active compounds).

6. Particularly understudied is the role of epibiotic biofilms for infection and disease of the host. When and how do biofilms repel pathogens and parasites, when do they stop doing this, when, and why do biofilm compounds switch from beneficial or neutral to pathogenic?
7. If certain strains are particularly beneficial for a host it would be of selective advantage if they were transmitted vertically on propagules or vegetative fragments, or horizontally on the surface of mesograzers. Attachment-detachment cycles permitting such hitchhiking and "contamination" have been described for other systems (Grossart et al., 2010).
8. Notwithstanding the general bias of this review in favor of the host-centered perspective it should be considered here that such vertical and horizontal transmission among conspecific hosts could benefit epibiotic strains by giving them a head start on a new substratum.
9. Widely neglected so far is the epibacterial perspective. Grossart and Tang (2010) have described how aggregation in biofilms affects bacterial ecology and evolution. But the specific significance of associating with a sessile host is understudied. In which regard do epibacteria benefit from host exudates, from the host-controlled conditions in the boundary layer and from the fact that they lead a stationary instead of a drifting way of life.
10. Finally, we recommend to progress from a compartmentalized view (host and epibacteria interacting with each other) to a more holistic view which recognizes that the holobiont (host with associated bacteria) is essentially inseparable physically and functionally and, perhaps, even evolutionarily.

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Ciliate epibionts associated with crustacean zooplankton in German lakes: distribution, motility, and bacterivory

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Ciliate epibionts associated with crustacean zooplankton are widespread in aquatic systems, but their ecological roles are little known. We studied the occurrence of ciliate epibionts on crustacean zooplankton in nine German lakes with different limnological features during the summer of 2011. We also measured the detachment and re-attachment rates of the ciliates, changes in their motility, and the feeding rates of attached vs. detached ciliate epibionts. Epibionts were found in all lakes sampled except an acidic lake with large humic inputs. Epibiont prevalence was as high as 80.96% on the cladoceran *Daphnia cucullata*, 67.17% on the cladoceran *Diaphanosoma brachyurum,* and 46.67% on the calanoid copepod *Eudiaptomus gracilis*. Both cladoceran groups typically had less than 10 epibionts per individual, while the epibiont load on *E. gracilis* ranged from 1 to >30 epibionts per individual. After the death of the zooplankton host, the peritrich ciliate epibiont *Epistylis* sp. detached in an exponential fashion with a half-life of 5 min, and 98% detached within 30 min, leaving behind the stalks used for attachment. Immediately after detachment, the ciliates were immotile, but 62% became motile within 60 min. When a new host was present, only 27% reattached after 120 min. The average measured ingestion rate and clearance rate of *Epistylis* were 11,745 bacteria ciliate^{-1} h^{-1} and 24.33 μl ciliate^{-1} h^{-1}, respectively. Despite their high feeding rates, relatively low epibiont abundances were observed in the field, which suggests either diversion of energy to stalk formation, high metabolic loss by the epibionts, or high mortality among the epibiont populations.

Keywords: ciliate epibionts, *Epistylis*, crustacean zooplankton, bacterivory, epibiont motility

INTRODUCTION

Both free-swimming and attached ciliates play key roles in freshwater and marine food webs (Sherr and Sherr, 1987; Sanders et al., 1989; Carrias et al., 1996). Although attached ciliates are very common in the benthos (Borror, 1968; Fenchel, 1969), their presence is not limited to bottom substrates. Many ciliates and other protozoans can attach themselves to various surfaces among the plankton such as suspended particles, phytoplankton, and zooplankton (Fernandez-Leborans and Tato-Porto, 2000; Christensen-Dalsgaard and Fenchel, 2003; Šimek et al., 2004). Attachment to a surface that experiences increased flow can enhance feeding rates by reducing the boundary layer surrounding the protozoan (Shimeta et al., 2001). In addition, because planktonic ciliates use their cilia both to generate thrust for swimming and to create a feeding current, attachment to a surface may help balance the thrust with drag and direct the flow field toward the cells, thereby increasing their food capturing efficiency relative to free-swimming individuals (Christensen-Dalsgaard and Fenchel, 2003). These predictions have been experimentally verified for flagellates (Christensen-Dalsgaard and Fenchel, 2003) and ciliates (Shimeta et al., 2001; Jonsson et al., 2004). Consequently, in systems where attached ciliates and other protozoans are abundant, they may contribute substantially to the total grazing impact. For example, attached flagellates on diatom colonies have been reported to account for up to 64% of all bacterivory by protists in an oligo-mesotrophic lake (Carrias et al., 1996) and a meso-eutrophic reservoir (Šimek et al., 2004).

While some ciliates may attach and detach in a haphazard manner that requires no special adhesive mechanism (Jonsson et al., 2004), others use distinct and elaborate structures, such as the stalks in peritrich ciliate epibionts, to more firmly attach to surfaces (Randall and Hopkins, 1962). The production of stalks and the subsequent loss of these structures during detachment represent a considerable energy investment by the ciliates, and must be compensated by substantial benefits of attachment. Based on previous studies with benthic ciliates (Shimeta et al., 2001) and stalk-less ciliate epibionts (Jonsson et al., 2004), one expects that stalked ciliates will have much higher feeding rates than their free-swimming form, although such a direct comparison is rarely made.

Within the aquatic environments, crustacean zooplankton such as copepods and cladocerans are the dominant members of the zooplankton community. Their exoskeleton provides abundant surfaces for attachment by a wide range of organisms, including bacteria, fungi, algae, and protozoans (Carman and Dobbs, 1997). Stalked ciliate epibionts on freshwater and marine crustacean zooplankton have been documented in many parts of the world (e.g., Fernandez-Leborans and Tato-Porto, 2000; Puckett

and Carman, 2002; Cabral et al., 2010; Rajabunizal and Ramanibai, 2011). Most studies tend to focus on the adverse effects these epibionts have on the host, such as decreased fecundity, interference with feeding, and locomotion, and increased sensitivity to contaminants (Kankaala and Eloranta, 1987; Puckett and Carman, 2002; Gilbert and Shröder, 2003). In comparison, quantitative information about the distribution and trophic impacts of these epibionts remains scarce (Utz and Coats, 2005a). Stalked epibiont ciliates exhibit two distinct life stages: the attached trophont stage for feeding, and the free-swimming telotroch stage for dispersal. Free-swimming telotrochs can result from asexual reproduction or through detachment and transformation of trophonts (Gilbert and Shröder, 2003). The transition from trophonts to telotrochs appears to be triggered by molting or death of the zooplankton host (Green, 1974; Willey and Threlkeld, 1995; Utz and Coats, 2008). Understanding the transition from attached to free-swimming form and vice versa, as well as the behaviors of each life stage, will help elucidate the life history and ecology of these organisms (Utz and Coats, 2008).

The northeastern region of Germany contains many glacially formed lakes with different nutrient conditions that are representative of lakes in other temperate regions. Many of them are important for inland fisheries, tourism, and navigation. While the occurrence of ciliate epibionts on crustacean zooplankton has been observed in some of these lakes (P. Kasprzak personal communication), no quantitative information is available. Our objective was to study the prevalence and abundance of ciliate epibionts attached to crustacean zooplankton in nine freshwater lakes with different limnological features, but located within a spatially small area in this region. Several of these lakes are part of a long-term monitoring program, and one of them, Lake Stechlin, is also a member of the international Global Lake Ecological Observatory Network. Additionally, we observed the behavior of the common peritrich ciliate epibiont, *Epistylis* sp., including its rate of detachment after host death, its motility after detachment, and its re-attachment to new hosts. We also measured rates of bacterivory by both attached and free-living stages of *Epistylis* sp. in laboratory experiments to estimate the potential importance of ciliate epibionts as bacterivores within these lakes.

MATERIALS AND METHODS

FIELD SAMPLING

Zooplankton samples were collected from nine lakes in Northeast Germany within a 20-km radius (**Figure 1**) over a 2-week period in July 2011. The lakes sampled were all glacially formed, and encompass a wide range of sizes, depths, and nutrient conditions (**Table 1**). Triplicate samples were collected from each lake by vertical tows with a 90-μm mesh plankton net equipped with a filtering cod end, immediately transferred to 100-ml glass jars, and preserved with Lugol's Iodine solution. Towed volumes were calculated based on the mouth diameter of the net (58 cm) and towed depths. In the laboratory, 5–15 ml subsamples were examined for zooplankton species composition, ciliate epibiont prevalence (the percent of a zooplankton group with ciliate epibionts), and epibiont load (the number of ciliate epibionts per individual zooplankter). Counts from the subsamples were extrapolated to the entire sample, and the average of the three replicates from each site was calculated. These values were used to estimate total epibiont densities (the number of epibionts per cubic meter) in each lake.

EPIBIONT DETACHMENT, MOTILITY, AND RE-ATTACHMENT OBSERVATIONS

While epibionts were found on a number of zooplankton species, the calanoid copepod *Eudiaptomus gracilis* with the ciliate epibiont *Epistylis* sp. was the most common in the samples, and was subsequently used for all laboratory experiments. *Eudiaptomus gracilis* was collected from Lake Dagow and transported back to the lab in ambient water. Copepods carrying large numbers of the ciliate epibiont *Epistylis* sp. were sorted into 5 μm-filtered surface lake water. Individual copepods were then transferred to a hanging drop slide with a small drop of the surrounding water. The copepod was killed by crushing its cephalosome with a fine-tipped forceps and the total number of epibionts initially attached to the copepod was immediately counted using a dissecting microscope. The number of epibionts that remained attached to the copepod carcass was counted every 5 min for up to 40 min after copepod death. Ten replicates were performed.

In a second experiment, copepods with attached *Epistylis* sp. were isolated and killed as described above. The copepod carcasses were removed after 30 min, and the motility of detached ciliates was observed every 20 min for 2 h. After the 2-h observation, a new copepod without epibionts was added to the ciliates. To allow the new copepod to move freely, the total volume was adjusted to 350 μl with 0.2 μm-filtered lake water. The copepod was visually inspected every 20 min and the number of reattached ciliates was estimated. After 2 h, the copepod was preserved with Lugol's Iodine solution and the actual number of attached ciliate epibionts was counted. Ten replicates were performed. All detachment, motility, and re-attachment observations were performed at room temperature (23°C).

BACTERIVORY EXPERIMENTS

The calanoid copepod *Eudiaptomus gracilis* and surface water were collected from Lake Stechlin and Lake Dagow, and transported back to the laboratory. In the laboratory, copepods without epibionts and copepods with a large number of epibionts were picked from the field samples and rinsed in 0.2 μm-filtered water from the same lakes to remove detritus. Ten millilitres of diluted lake water (1:50 and 1:100 for Lake Stechlin and Lake Dagow, respectively) were added to 25-ml sterilized glass vials. Dilutions were prepared by adding 5 μm-filtered lake water to 0.2 μm-filtered lake water. Four sets of vials, each set with five replicates, were prepared as follows: (1) vials for enumerating initial bacterial abundance, (2) control vials without copepods to account for bacterial growth during the incubation, (3) experimental vials for copepods without epibionts, and (4) experimental vials for copepods with epibionts. Three copepods were gently pipetted into each of the experimental vials, with minimal surrounding water. Copepod addition was mimicked in the initial and control vials. Vials for initial bacterial abundance were processed immediately; all other vials were incubated for 2 h at room temperature (23°C). To measure free-living bacterial abundance, vials were swirled gently to mix the water; a 2-ml aliquot was removed without removing the copepods, and filtered onto a 0.2 μm polycarbonate membrane

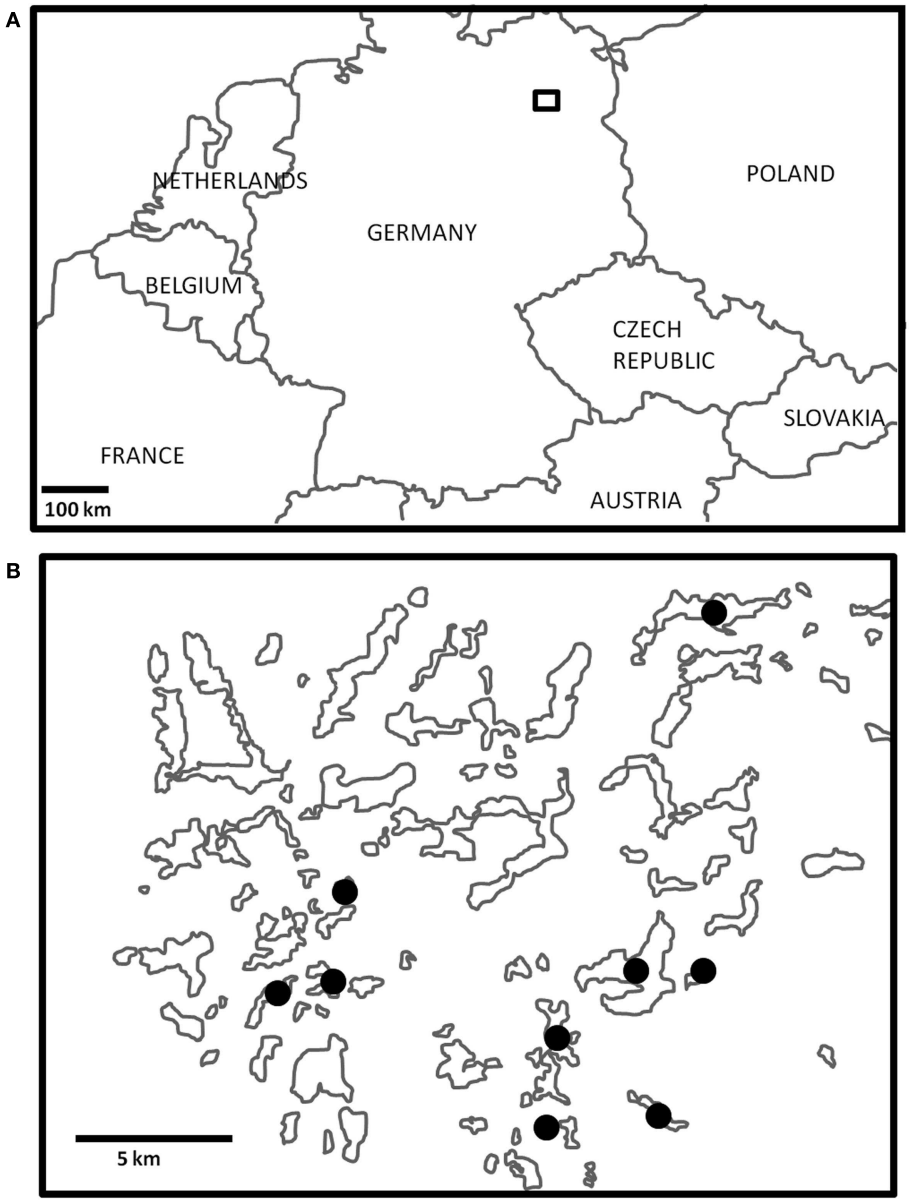

FIGURE 1 | (A) The general study region in northeast Germany is outlined by the small square box, which is enlarged in **(B)**. Three Lakes sampled are denoted by black dots.

filter. Samples were stained with SYBR-gold nucleic acid stain in Citifluor and counted on an epifluorescent microscope with blue light excitation. Vials containing copepods were preserved with Lugol's iodine solution and the total number of ciliate epibionts in each vial was counted.

To measure the grazing rates of free-swimming ciliate epibionts, copepods carrying *Epistylis* sp. were collected from Lake Dagow and sorted into 0.2 μm-filtered surface lake water. Individual copepods were killed as described previously, and the ciliates were allowed to detach for 30 min. Afterward, the copepod carcass was removed, and 50–56 free-swimming ciliates were transferred to each of the experimental vials containing 10 ml of diluted Lake Dagow water, prepared as described previously. Three sets of vials, each set with five replicates, were established as follows: (1) vials for initial bacterial abundance, (2) control vials without ciliates, and (3) experimental vials with free-swimming ciliates. The vials were incubated at room temperature for 2 h. Bacterial abundance was measured as described previously. Bacterial abundance data were tested for normality and differences among the treatments were assessed with a one-way ANOVA followed by *post hoc* Tukey pairwise comparisons. When a significant change in bacterial abundance was observed, ingestion and clearance rates were calculated after correcting for changes in bacterial abundances in the control and epibiont-free copepod treatments.

Table 1 | Limnological characteristics for sampled lakes.

Lake	Area (km^2)	Max depth (m)	Nutrient condition	Latitude and longitude
Dagow	0.22	8	Eutrophic	53°09′N, 13°03′E
Stechlin	4.30	68	Oligotrophic	53°09′N, 13°01′E
Grosse Fuchskuhle	0.08	5.6	Eutrophic	53°10′N, 13°02′E
Nehmitz	1.60	18.6	Mesotrophic	53°08′N, 12°59′E
Roofen	0.57	19.1	Mesotrophic	53°06′N, 13°02′E
Drewen	2.56	9	Eutrophic	53°15′N, 13°03′E
Prebelow	2.80	7.6	Eutrophic	53°10′N, 12°52′E
Schlaborn	0.70	9	Eutrophic	53°09′N, 12°52′E
Dollgow	0.18	2	Eutrophic	53°04′N, 13°00′E

RESULTS

FIELD STUDY

The most common zooplankton species found among the lakes were the calanoid copepod *Eudiaptomus gracilis* and the cladocerans *Daphnia cucullata* and *Diaphanosoma brachyurum* (**Table 2**). Cyclopoid copepods were also present, but were not identified to any lower taxonomic level. A variety of epibionts, including diatoms and ciliates, were found in association with zooplankton in the lakes. Ciliate epibionts were identified according to criteria outlined by Patterson and Hedley (2003). The mobile peritrich ciliate *Trichodina* sp. was commonly observed on live copepods from Lake Dagow and Lake Stechlin; however, it immediately detached upon preservation, and therefore was not included in the data. The stalked peritrich ciliate *Vorticella* sp. was occasionally observed on cladocerans. The most common epibiont carried by *E. gracilis*, *D. cucullata*, and *D. brachyurum* was identified as the peritrich ciliate *Epistylis* sp. based on the stalk branching pattern, stalk thickness relative to cell size, and the non-contractile nature of the stalks (**Figures 2A–D**). Cyclopoid copepods occasionally carried *Epistylis* sp., but the majority of epibionts carried by cyclopoids were peritrich ciliates belonging to the family Opercularidae (**Figure 2E**). The prevalence of epibionts was highly variable within a single species among the different lakes, and among different species within the same lake. Among the sampled lakes, epibiont prevalence on *E. gracilis* ranged from 0% (Lake Dollgow) to 46.67% (Lake Stechlin) of the population (**Figure 3**). Epibiont prevalence ranged between 0% (Lakes Stechlin and Nehmitz) and 80.96% (Lake Dagow) on *D. cucullata* and between 0% (Lake Stechlin) and 67.16% (Lake Dagow) on *D. brachyurum* (**Figure 3**). Epibionts were found on at least one zooplankton group in all lakes sampled except Lake Grosse Fuchskuhle.

Of the various zooplankton species where at least 5% of the population carried one or more epibionts, species-specific frequency distributions of epibiont load were noted. The epibiont load on *E. gracilis* ranged from 1 to >30, and the frequency distribution was fairly uniform in each of the lakes (**Figure 4**). The two cladoceran species, *D. cucullata* and *D. brachyurum*, showed a similar range in epibiont load (1 to >30); however, the frequency distribution was skewed toward the lower end such that the majority of cladocerans carried ≤15 epibionts per individual (**Figure 4**). The epibiont loads on cyclopoid copepods were also fairly uniformly distributed, with the exception of Lake Drewen, in which 50.02% of cyclopoids carried >30 epibionts per individual (**Figure 4**). The estimated densities of epibionts in the lakes are presented in **Table 2**.

EPIBIONT DETACHMENT, MOTILITY, AND RE-ATTACHMENT

After the death of the copepod host, 49.4% of all epibionts detached within the first 5 min. During detachment the ciliate exhibited a rocking motion to break free from the stalk, leaving the stalk attached to the copepod host (**Figures 2C,D**). Immediately after detachment, the ciliates were either immobile or swimming very slowly in tight circular patterns. After 30 min, the copepod carcass was removed; 98.1% of the epibionts had detached (**Figure 5A**) and observations of their motility continued. The detachment of epibionts from copepod carcasses was well described ($R^2 = 0.998$, $p < 0.0001$) by the exponential decay function:

$$y_1 = 99.7e^{-0.136x}$$

where y_1 is percent of epibionts that remained on the copepod and x is time in minutes. Twenty minutes after the removal of copepod carcass (i.e., 50 min. after host death), only 1.28% of the detached ciliates were active and rapidly swimming. Motility increased to 62.14% by 60 min after carcass removal, and 90.18% by 120 min after carcass removal (**Figure 5B**). The percentage of detached ciliates actively swimming (y_2) as a function of time (x; minutes) could be described ($R^2 = 0.986$, $p = 0.0027$) by the polynomial equation:

$$y_2 = -2.811 + 0.1561x + 0.0231x^2 - 0.0002x^3$$

When a new copepod host was introduced, 20.13% of the free-swimming ciliates attached to the new host within the first 20 min, but re-attachment leveled off at 27.27% after 120 min (**Figure 5C**). The percentage of reattached ciliates (y_3) as a function of time (x) was well represented ($R^2 = 0.995$, $p < 0.0001$) by the equation:

$$y_3 = 26.83(1 - e^{-0.65x})$$

BACTERIVORY RATES

In the first experiment conducted with the copepod *E. gracilis*, there was a significant increase in bacterial abundance in both the control vials and copepod vials ($p < 0.001$), but the final bacterial abundance was significantly lower in vials

Table 2 | Mean (SD) densities of common zooplankton species and their associated ciliate epibionts in the lakes studied.

		Lake											
		Dagow	Stechlin	Grosse Fuchskuhle				Nehmitz	Roofen	Drewen	Grosser Prebelow	Schlaborn	Dollgow
				SE	NE	NW	SW						
DENSITY: INDIVIDUALS m^{-3} (MEAN SD)													
E. gracilis	Zooplankter	2656 (418)	392 (61)	18,542 (3278)	10,168 (3780)	10,713 (2577)	0(0)	1972 (145)	977 (132)	382 (10)	492 (157)	522 (206)	0 (0)
	Epibiont	56,092 (77,605)	2823 (1067)	0 (0)	0 (0)	0 (0)	0(0)	1031 (722)	990 (1057)	144 (215)	968 (430)	1018 (155)	0 (0)
D. cucullata	Zooplankter	1937 (641)	1129 (272)	0 (0)	0 (0)	0 (0)	0(0)	668 (45)	1368 (214)	778 (181)	1077 (226)	2327 (382)	686(172)
	Epibiont	17,303 (5252)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	0 (0)	348 (149)	1173 (504)	559 (227)	540 (115)	381(449)
D. brachyurum	Zooplankter	1169 (111)	109 (53)	0 (0)	0 (0)	0 (0)	0(0)	2235 (589)	579 (112)	1197 (269)	2528 (205)	782 (98)	398(87)
	Epibiont	642,726 (258,241)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	22 (38)	103 (90)	2776 (904)	1444 (664)	430 (146)	1130(570)
Cyclopoid	Zooplankter	nc	nc	0 (0)	0 (0)	0 (0)	0(0)	255 (220)	919 (56)	2140 (76)	3009 (853)	2185 (411)	2349(1088)
	Epibiont	nc	nc	0 (0)	0 (0)	0 (0)	0(0)	0 (0)	115 (199)	38,193 (13,257)	7261 (3812)	12 (21)	8508(3462)
Bosmina	Zooplankter	nc	nc	0 (0)	0 (0)	0 (0)	0 (0)	942 (152)	696 (157)	106 (37)	1208 (214)	1903 (204)	170(27)
	Epibiont	nc	nc	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	55 (95)	188 (32)	0 (0)	0 (0)
Ceriodaphnia	Zooplankter	nc	nc	9297 (3918)	6388 (2798)	11,394 (3889)	53,752 (9326)	77 (53)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Epibiont	nc	nc	0 (0)	0 (0)	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
EPIBIONT BACTERIVORY													
(10^8) Bacteria consumed (m^{-3} day^{-1})		75.2	8.4	0	0	0	0	3.37	4.77	122	34.1	6.51	30.1
% Bacterial standing stock consumed		0.189	0.021	0	0	0	0	0.008	0.012	0.304	0.085	0.016	0.075

Lake Grosse Fuchskuhle is divided into four basins (SE, NE, NW, and SW). The estimated number of bacteria grazed by all ciliate epibionts and the percentage of bacterial standing stock consumed by ciliate epibionts within each lake are also presented. For bacterivory calculations, the ingestion rate used was the average between Lake Stechlin and Lake Dagow. An ambient bacterial abundance of 4×10^6 cells ml^{-1} was assumed.

Epibiont = ciliate epibionts associated with that particular zooplankter per m^{-3}.

nc = not counted.

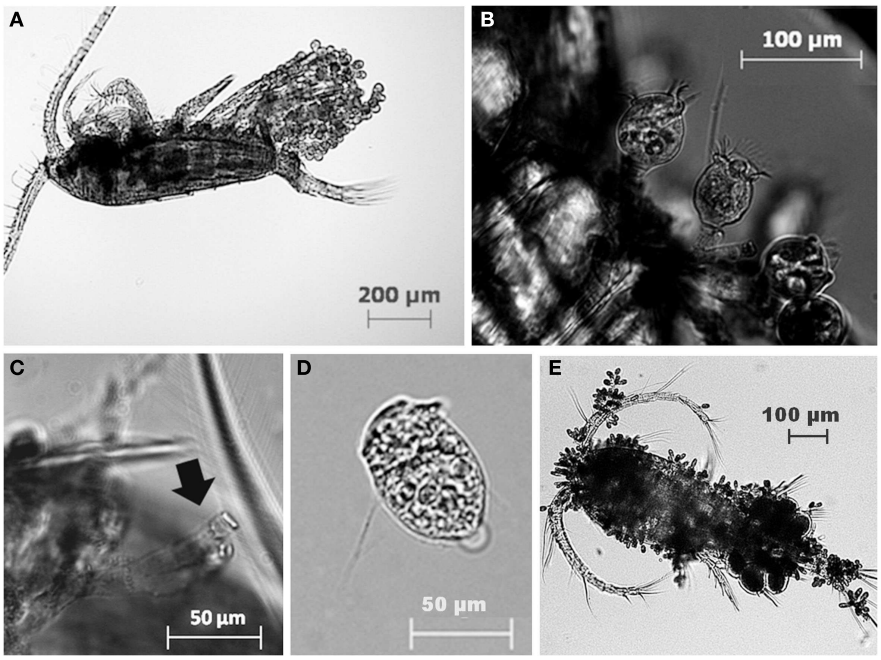

FIGURE 2 | Examples of the peritrich ciliate epibiont *Epistylis* sp. attached to the calanoid copepod *Eudiaptomus gracilis* (A,B) and the residual stalk (C) left attached to the dead copepod after detachment (D). Peritrich ciliates of the family Opercularidae, attached to a cyclopoid copepod **(E)**.

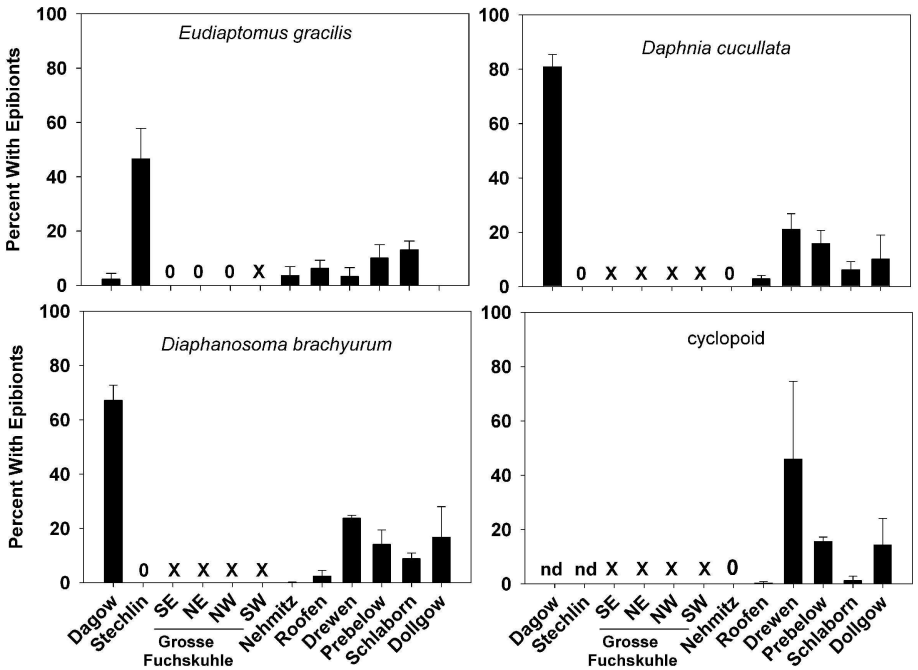

FIGURE 3 | Prevalence of ciliate epibionts (percent of the zooplankton population with one or more ciliate epibionts; mean ± SD, *n* = 3) on common zooplankters *Eudiaptomus gracilis, Daphnia cucullata, Diaphanosoma brachyurum,* and Cyclopoid copepods in each lake. 0 = no epibionts were found on that zooplankton group, X = zooplankton group was not present in that lake, nd = no data.

with copepods that carried attached epibionts ($p < 0.001$), indicating grazing activities by the epibionts. Ingestion rates were 11,745 ± 6701 and 11,065 ± 2986 (mean ± SD) bacteria ciliate^{-1} h^{-1} for Lake Stechlin and Lake Dagow, respectively (**Figure 6**). The corresponding clearance rates were 24.33 ± 13.88 and 9.49 ± 2.56 (mean ± SD) µl ciliate^{-1} h^{-1}, respectively. In

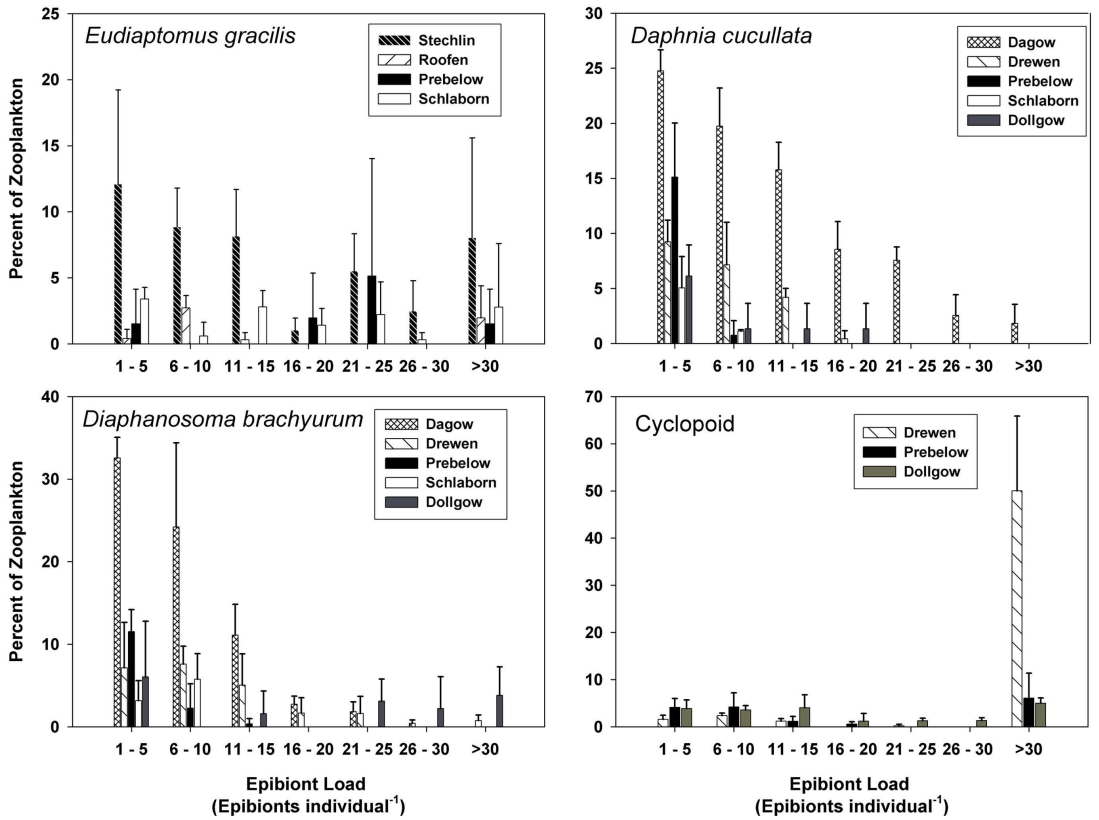

FIGURE 4 | Frequency distribution of epibiont load (number of epibionts per individual, mean ± SD, *n* = 3) on *E. gracilis, D. cucullata, D. brachyurum,* and cyclopoid copepods. Epibiont load is presented only for lakes where epibionts were found on >5% of the zooplankton populations.

the second experiment conducted with free-swimming ciliate epibionts, there was no significant change in the bacterial abundance in either the control vials or vials with ciliates, indicating that grazing activity by free-swimming epibionts was negligible.

DISCUSSION

EPIBIONT ABUNDANCE AND DISTRIBUTION

The peritrich ciliate *Epistylis* sp. observed in our study exhibited a low degree of host specificity and colonized both calanoid copepods and cladocerans, consistent with other studies (Green, 1974; López et al., 1998; Gilbert and Shröder, 2003). Despite the close proximity and sampling period of the different lakes, there were large inexplicable differences in the prevalence of epibionts between the different zooplankton species within the same lake, and between the different lakes for the same zooplankton species. For example, both cladocerans *D. cucullata* and *D. brachyurum* were heavily infested (>60% of the population) in Lake Dagow, but no epibionts were found on these species in Lake Stechlin. In contrast, 46% of the copepod *E. gracilis* carried epibionts in Lake Stechlin but less than 5% had epibionts in Lake Dagow. Because both cladocerans and copepods are covered by chitinous carapace, it is unlikely that the different prevalence was a result of differences in body surface chemistry. In laboratory experiments, Gilbert and Shröder (2003) showed that *Epistylis pygmaeum* preferentially attached to some zooplankton species but not others, but the cause remained unknown. Although Lake Dagow and Lake Stechlin are physically connected by a small drainage canal, they are very different environments: Lake Dagow is a shallow, eutrophic lake while Lake Stechlin is a deep, oligotrophic lake. The different environmental conditions between the lakes may modulate epibiont attachment to the different zooplankton species (Threlkeld et al., 1993).

Overall, the ciliate epibiont prevalence observed in our study was similar to that reported in other field studies. For examples, approximately 35% of Chironomid larvae carried the ciliate epibiont *Rhabdostyla* in a tropical lake system (Cabral et al., 2010), 48% of the calanoid copepod *Metridia pacifica* collected from the northeast Pacific Ocean were infested with suctorian ciliates (Ohtsuka et al., 2011), 50% of the Harpactacoid copepod *Coullana* spp. from a salt marsh carried peritrich ciliates (Puckett and Carman, 2002), and up to 87% of rotifers maintained in culture supported *Epistylis* sp. (Gilbert and Shröder, 2003). In our study, Lake Grosse Fuchskuhle was the only lake in which no epibionts were found on any zooplankton species. Lake Grosse Fuchskuhle is an acidic lake with large humic inputs (average pH = 4.2–4.6; Casper et al., 2003), whereas the average pH in the other lakes is slightly basic (e.g., average pH during the study period = 8.1 in Lake Dagow and Lake Stechlin). This suggests that the acidic environment in Lake Grosse Fuchskuhle is unfavorable to the epibionts.

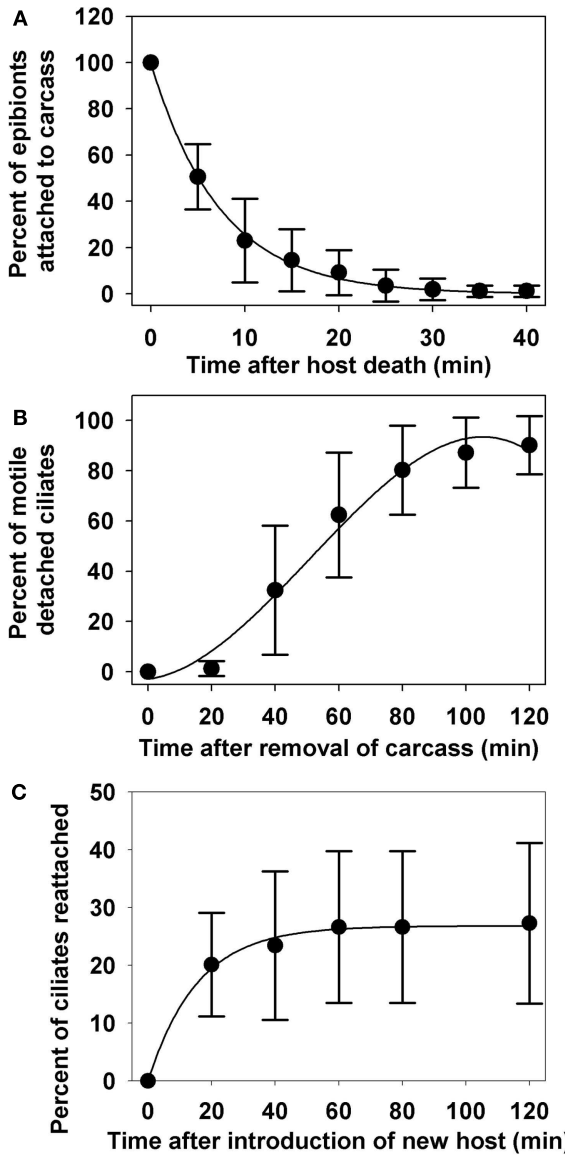

FIGURE 5 | Percentage of epibionts remaining on zooplankton host, *E. gracilis* after host death (A), motility of epibionts detached from copepod carcasses (B), and re-attachment of free-swimming ciliates to new copepod hosts (C). All data points are mean values ± SD ($n = 10$).

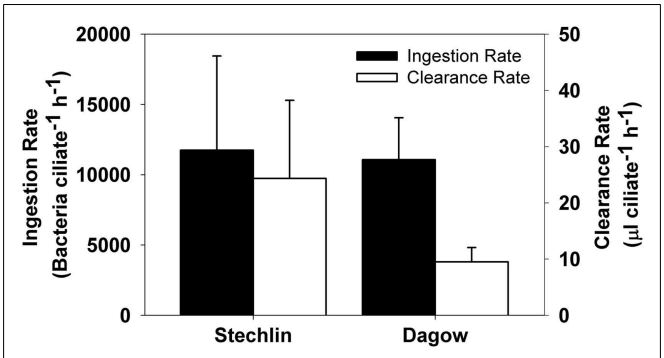

FIGURE 6 | Estimated ingestion rates (black bars, left axis) and clearance rates (white bars, right axis) of the peritrich ciliate *Epistylis* sp. when attached to the calanoid copepod *Eudiaptomus gracilis* collected from Lake Stechlin and Lake Dagow (mean ± SD, $n = 5$).

EPIBIONT BEHAVIOR

The rapid detachment after death of the zooplankton host suggests that the ciliate epibiont was able to quickly detect a change in the host condition that made attachment no longer beneficial. Utz and Coats (2005b) suggested that some chemical or electrical cue may travel through the stalk of the epibiont to the zooid. Changes in these cues may occur during molting or after death, and trigger detachment. In this study, the number of epibionts attached to *E. gracilis* decreased exponentially after host death. This same behavior was observed in the marine ciliate epibiont *Zoothamnium intermedium* detaching from the calanoid copepods *Acartia tonsa* and *Eurytemora affinis*, but at different rates (Utz and Coats, 2008). The epibionts in our study had a detachment "half-life" of 5 min; i.e., approximately 50% of the epibionts detached within 5 min of host death and 98% detached within 30 min. In contrast, *Z. intermedium* detached at a much lower rate: 50 and 90% after 3 and 7 h after host death, respectively (Utz and Coats, 2008). Even chemically induced detachment occurred over a longer time period, with 80% of *Vorticella* detaching within 90 min (de Baufer et al., 1999). Re-attachment of the free-swimming ciliates to a new host also occurred on a shorter time scale in our experiments: 27% of the free-swimming ciliates attached within 2 h of introduction of a new host. In comparison, only ∼15% of *Z. intermedium* attached within 2 h (Utz and Coats, 2008). The different detachment and re-attachment rates may reflect the extent to which the different ciliate species have adapted to a benthic life style among the plankton.

After detachment, the ciliates initially showed no or little movement, and only gradually regained full motility (**Figure 5B**). Gilbert and Shröder (2003) observed two distinct swimming patterns among different life stages of free-swimming *E. pygmaeum*: (i) detached zooids swimming slowly in a circular pattern, and (ii) fast, erratic-swimming telotrochs; they suggested that the *Epistylis* was able to switch back and forth between the two forms. Both swimming patterns were noted in our study, with a clear delay between the occurrence of the two different motilities, which may be a result of the time needed for physiological changes during the transformation from attached zooid to free-living zooid and/or telotroch. It has been suggested that the fast-swimming telotroch form does not feed and primarily functions as a way to find new hosts, whereas the slower, free-swimming zooid can feed and reproduce, allowing populations to persist when hosts are rare (Gilbert and Shröder, 2003).

The difference between detachment rate and re-attachment rate in our study is quite striking. While almost all epibionts detached within 40 min, less than 30% of them reattached even after 2 h (**Figures 5A,C**). Re-attachment was not limited by encounter probability because in our experiments, a new copepod host was exposed to an average ciliate density of 86 ciliates ml^{-1}, and physical contact between copepod and free-swimming ciliates occurred frequently. However, most instances of physical contact did not

result in attachment. Gilbert and Shröder (2003) suggested that attachment of *E. pygmaeum* is mediated by contact recognition of surface properties, which may be different on different body parts of the copepod. Local, small-scale water movement, such as the copepod feeding current, may also alter attachment efficiency.

Attachment is an adaptive behavior that can result in increased feeding rates (Christensen-Dalsgaard and Fenchel, 2003; Jonsson et al., 2004). However, attachment also has disadvantages because it restricts the movement of the ciliate, and exposes the ciliate to the same predation risks experienced by the host, or even increases predation risk of the host (e.g., Willey et al., 1990). For stalked epibionts such as the *Epistylis* species used in this study, more permanent attachment represents a significant energy investment in the form of stalk production. Using a stalk for attachment also further restricts the ciliate's movement, putting it at an even greater risk than other more mobile epibionts, such as *Trichodina sp.* From a cost vs. benefit perspective, it is perhaps not surprising that re-attachment rate should be lower than detachment rate, as the ciliate would have to carefully select the right location for attachment, but it should quickly abandon the host under unfavorable condition (e.g., death of the host). The considerable energy investment for stalk production and the risks associated with attachment also implies that the ciliate must be able to extract substantial benefits for being attached. One such benefit is enhanced feeding efficiency as demonstrated in our bacterivory experiments.

BACTERIVORY RATES AND FEEDING IMPACTS

Significant feeding rates of the epibiont were measurable for the attached form, but not for the detached, free-swimming form. The short incubation time in our feeding experiments may have limited the feeding signal that we could detect with the free-swimming ciliates; nonetheless, our results indicate that the attached form had a much higher feeding efficiency than the free-swimming form of the epibiont. The estimated clearance rates of the attached epibiont were 9.49 ± 2.56 and 24.33 ± 13.88 (mean $\pm$ SD) μl ciliate^{-1} h^{-1} for Lake Dagow and Lake Stechlin, respectively. These were much higher than an expected clearance rate of $\sim$0.5 μl ciliate^{-1} h^{-1} for free-living ciliates of similar size (ca. 45 μm equivalent spherical diameter (ESD; Fenchel, 1980). They were also higher than the reported maximum clearance rate of an *Epistylis* colony measured with monodisperse fluorescent latex beads (1.25 μl ciliate^{-1} h^{-1}; Børsheim, 1984). However, the use of latex beads tends to underestimate protozoan feeding rate (Sherr et al., 1987), and a similarly high clearance rate has been observed for some ciliate species, e.g., *Lohmanniella spiralis* with a clearance rate of 24 μl ciliate^{-1} h^{-1} (Jonsson, 1986). The higher clearance rate of the attached form relative to the free-swimming form provides a clear benefit for epibiont attachment. The kinetics of small-scale water movement around a copepod and its abilities to deliver food and oxygen are an important factor in determining the distribution of ciliate epibionts on the copepod (Fernandez-Leborans et al., 2006). A marine benthic ciliate community can take advantage of the reduced boundary layer due to increased flow over the sediment surface and increase its clearance rate by a factor of 5 (Shimeta et al., 2001). The same concept can be applied to ciliate epibionts on zooplankton: As a zooplankter swims through the water, flow over the body surface increases, reducing the boundary layer, and allowing the epibionts to feed at a higher rate.

The estimated average ingestion rate of attached epibionts in our experiments was 11,745 bacteria ciliate^{-1} h^{-1}, which is 1–3 orders of magnitude higher than that of common free-living bacterivorous ciliates (Sanders et al., 1989). Using fluorescent beads to investigate the ingestion rate of *Epistylis* within wastewater biofilms, Eisenmann et al. (2001) reported an ingestion rate as high as 1200 beads ciliate^{-1} h^{-1}. Methodological differences may contribute to the difference in ingestion rate between our study and that by Eisenmann et al. (2001): The fluorescent bead technique measures what is actually ingested by the ciliates, whereas our incubation experiments measured what disappeared from the surrounding water. Increased ciliate filtration may have brought the bacteria in closer proximity to the copepod, where they could have attached to the copepod or ciliate stalks, thereby causing an overestimation of ingestion rates in our experiments. On the other hand, considering that the use of fluorescently labeled beads could underestimate bacterivory by a factor of 10 (Sherr et al., 1987), our measured ingestion rates were indeed comparable to that for *Epistylis* biofilms (Eisenmann et al., 2001).

Studies on the grazing impact of planktonic ciliates are often focused on free-living species (e.g., Fenchel, 1980), whereas the grazing impact of ciliate epibionts attached to zooplankton is rarely measured. Using our measured clearance rates and epibiont abundances for the various lakes, we estimated that bacterivory by zooplankton-associated ciliate epibionts in these lakes removes only 0.3% of the bacterial biomass present in the water column per day (**Table 2**). This small impact is a result of the low epibiont abundances within these lakes. Despite the low overall trophic impact, selective feeding by epibionts may influence the free-living bacterial community composition. Additionally, the high individual clearance rate and ingestion rate of the epibionts mean that each ciliate processed a large amount of bacterial biomass. Utz (2008) reported that at a food concentration of 10^6 bacteria ml^{-1}, comparable to our experimental food concentrations, the epibiont ciliate *Zoothamnium intermedium* attained a growth rate as high as 0.8 day^{-1}. An even higher *in situ* growth rate (1.37 day^{-1}) has been reported for benthic peritrich ciliate (Kusuoka and Watanabe, 1987). Our short incubation time did not allow us to measure growth. Nevertheless, if *Epistylis* sp. grows at a comparable rate in our lakes, its low *in situ* abundances would imply high mortality (Kusuoka and Watanabe, 1987). On the other hand, Sherr and Sherr (2002) calculated that a ciliate with an ESD of 40 μm feeding at a food concentration of 10 μg C L^{-1} would require a clearance rate of 20 μl cell^{-1} h^{-1} to support one cell doubling per day. The epibionts in our experiments had an ESD of 40–50 μm (excluding the stalk). Assuming a bacterial carbon content of 12.4 fg per cell (Fukuda et al., 1998), the food concentration in our experiments would be approximately 3.72 μg C L^{-1}, which is less than the theoretical amount required to sustain one doubling per day. For stalked ciliates, growth may also manifest as stalk production in addition to cell multiplication. As the epibiont colony grows, the proportion of stalk biomass also increases. Using digitized images, we estimated that the biovolume ratio between the stalk and the cell ranged from 0.0097 for recently attached *Epistylis* to 0.44 for

larger *Epistylis* colonies with elaborate stalk structures, and the average stalk-to-cell biovolume ratio was 0.18. As the stalk structure grows, it moves the ciliate farther away from the copepod surface, which may help increase feeding efficiency by reducing the formation of small-scale eddies near the surface (Pepper et al., 2010).

Alternative to being used for biomass production, ingested energy could be lost through respiration and excretion. The gross growth efficiency of free-living ciliates is ca. 0.4 (Hansen et al., 1997), hence, on average 60% of the ingested materials is respired or excreted. Assuming that the epibionts in our experiments had the same gross growth efficiency, their high feeding rate would translate to high respiration and excretion rates, making these epibiont colonies potential "hotspots" for remineralization within the water column.

In summary, epibiont ciliates were prevalent in all of the studied lakes except the acidic Lake Grosse Fuchskuhle. The high individual feeding rates but low abundances of the epibionts suggest that the epibiont populations were either controlled by high mortality or that a high percentage of the ingested bacterial biomass was remineralized. It is also possible that selective feeding by the epibionts may influence the free-living bacterial community in quality rather than quantity. Additional research into the population dynamics, grazing and bioenergetics of the epibionts will help resolve these questions and provide further insights into the ecological roles of these epibiont ciliates.

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Act together—implications of symbioses in aquatic ciliates

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Mutual interactions in the form of symbioses can increase the fitness of organisms and provide them with the capacity to occupy new ecological niches. The formation of obligate symbioses allows for rapid evolution of new life forms including multitrophic consortia. Microbes are important components of many known endosymbioses and their short generation times and strong potential for genetic exchange may be important drivers of speciation. Hosts provide endo- and ectosymbionts with stable, nutrient-rich environments, and protection from grazers. This is of particular importance in aquatic ecosystems, which are often highly variable, harsh, and nutrient-deficient habitats. It is therefore not surprising that symbioses are widespread in both marine and freshwater environments. Symbioses in aquatic ciliates are good model systems for exploring symbiont-host interactions. Many ciliate species are globally distributed and have been intensively studied in the context of plastid evolution. Their relatively large cell size offers an ideal habitat for numerous microorganisms with different functional traits including commensalism and parasitism. Phagocytosis facilitates the formation of symbiotic relationships, particularly since some ingested microorganisms can escape the digestion. For example, photoautotrophic algae and methanogens represent endosymbionts that greatly extend the biogeochemical functions of their hosts. Consequently, symbiotic relationships between protists and prokaryotes are widespread and often result in new ecological functions of the symbiotic communities. This enables ciliates to thrive under a wide range of environmental conditions including ultraoligotrophic or anoxic habitats. We summarize the current understanding of this exciting research topic to identify the many areas in which knowledge is lacking and to stimulate future research by providing an overview on new methodologies and by formulating a number of emerging questions in this field.

Keywords: aquatic ciliates, ciliate-bacteria interaction, symbiosis, associated prokaryotes, microbial functions, ecosystem function

INTRODUCTION

Ciliates are an extraordinarily widespread group of protists that occur in almost all aquatic environments. These include coastal waters, hydrothermal vents, anoxic sediments, hyporheic zones, and oxic as well as anoxic parts of the water column. Ciliates are very abundant phagotrophs in the biosphere and are capable of forming extensive blooms. They are important grazers of algae, bacteria, and other microorganisms (Taylor and Sullivan, 1984; Sherr and Sherr, 1987; Eppstein, 1997). They promote the re-mineralization of microbial biomass and increase the transfer of nutrients to other organisms (Vickerman, 1992). The global diversity of free-living ciliates is surprisingly low (ca. 3000; Finlay et al., 1998), but this number has been called into question. Foissner and colleagues (2007) calculated that there are between 27,000 and 40,000 free-living ciliate species by using the moderate endemicity model. An estimation of ciliate species diversity based on small-subunit ribosomal sequences seems to be unreliable (Nebel et al., 2011) due to the fact that sequence similarity among different species can vary widely (see Caron et al., 2012).

Endosymbiosis may represent a general evolutionary strategy by which phagotrophic protists acquire novel metabolic functions such as photosynthesis, nitrogen fixation and recycling, methanogenesis, and sulphide oxidation. They can therefore be regarded as an important source of genetic innovation (Nowack and Melkonian, 2010). Protists harbor bacteria, algae, fungi, and viruses (Gibson, 1974) and are regarded as popular symbioses initiators with other microorganisms, particularly bacteria (Soldo, 1987). Such symbioses may be more widespread among protists than previously thought (Gast et al., 2009). In ciliates, both endosymbionts and ectosymbionts have been repeatedly reported in aerobic and anaerobic environments (Rosati, 2004). It is thought that ciliates had a photosynthetic ancestor (Reyes-Prieto et al., 2008) but lost their plastids. This would decrease the number of required symbioses for the development of plastids

(Archibald, 2008). Today, ciliates host plastids as well as other ecto- and endosymbionts, a fact known since the late 19th century.

Despite their early discovery, the diversity and ecological function of ciliate symbioses are surprisingly little understood. Here we attempt to summarize symbiotic interactions with ciliates and their ecological implications for both freshwater and marine ecosystems. We also highlight a number of emerging research questions regarding the dynamics and ecological traits of symbioses between the ciliate host and its prokaryotic symbionts. We maintain that these model organisms may enlighten more general processes of establishment and maintenance of symbiosis.

AN OVERVIEW OF SYMBIOSES

We use here symbiosis sensu De Bary (1879) who defined it as long-term interactions between two different organisms. Symbionts may affect their hosts in a positive or negative manner (**Table 1**). They have the potential to significantly affect the ecology, physiology, and evolution of both partners (Cavanaugh et al., 2006; Gast et al., 2009) and may therefore facilitate the occupation of new ecological niches and have an impact on whole food webs and ecosystems (Görtz and Brigge, 1998; **Table 2**). Symbionts and hosts represent consortia with two or more coexisting and interacting genomes. These form metabolic competencies and natural selection therefore operates on these integrated consortia (Finlay, 2004).

Symbioses can be mutualistic, commensalistic, or parasitic. Most symbioses investigated to date are mutualistic. Common examples include symbioses with photoautotrophs, lithoautotrophic prokaryotes, and organoheterotrophic bacteria. Mutualism benefits both partners by nutrient and energy supply, and through protection from predators or environmental threats such as oxygen radicals and toxins (Boucher et al., 1982). Many mutualistic microbial symbioses have a biochemical origin, based on the transfer of compounds produced by one partner or the other (Hoffmeister and Martin, 2003). Mutualistic symbioses increase the metabolic competencies of such consortia and enable these entities to colonize otherwise inaccessible habitats (Cavanaugh et al., 2006; Kleiner et al., 2012). However, benefits of symbionts for their hosts may vary with ecological conditions or life-stages (Polz et al., 2000; Hay et al., 2004) and are variable in time. In addition, these consortia do not implicitly suggest mutual benefits, but can be sometimes regarded as a trap for both partners, with no chance to escape the relationship. In such a case it will be interesting to study how they can cope as an entity in a new ecological niche and/or compete with uninfected individuals of the host's species (Görtz and Fokin, 2009).

Although most studies on symbioses focus on intracellular microbes, the first step in evolutionary development of eukaryotes may have been the formation of consortia with ectosymbionts offering protection against environmental and physiological hazards (Rosati, 2004). Symbioses, and endosymbioses in particular, drastically accelerate evolutionary changes in genomes of the symbiotic partners, which can be seen as the result of physical proximity and growing liaison of completely different organisms (Shinzato and Kamagata, 2010). Thereby, the establishment of symbiotic interactions typically seems to be established due to specific metabolic capabilities of the symbionts (Hoffmeister and Martin, 2003; Nowack and Melkonian, 2010). In eutrophic, stable environments, however, endosymbioses could be more based on

Table 2 | Potential and *published* ecological consequences of symbioses in aquatic ciliates (for references see text).

	Effects of the entity host-symbiont
Material cycling	• ***High photosynthesis rates***
	• ***Methanogenesis***
	• ***Sulfur transformation***
	• ***Demands on iron and magnesium***
	• Nitrogen fixation
	• Phosphate storage
Other organisms	• ***Competitive advantage***
	• ***Toxin production***
	• Reservoir and vector for pathogens
Ecosystem	• ***Covering new ecological niches***
	• Higher autochthon biomass formation
	• Strengthening of the microbial loop

Table 1 | Effects of symbiosis on ciliate hosts and symbionts.

	Effects on host	Effects on symbiont
Positive	• Supply of nutrients and organic matter, growth factors, vitamins etc.	• Nutrient supply
	• Competitive advantage	• movement to favorite conditions, increased motility
	• Protection against parasites	• grazing protection
	• Oxygen removal by heterotrophs	• disposal of organic or inorganic material
	• Degradation of metabolic waste; detoxification	• Less competitors
	• Protection against UV radiation	• Supply with CO_2 and H_2
	• Adaptation of aerobic life in anoxic zones	
Neutral or unclear	• Space requirements by symbionts	• Better genetic exchange?
	• H_2 scavengers in anaerobic ciliates (backup for methanogens?)	• Constant conditions, e.g., pH?
	• Energy transfer?	
	• Higher grazing pressure?	
negative	• Competition for nutrients and organic matter	• genetic bottleneck effect
	• cell lysis	• digestion by the host
	• inhibition by toxins etc.	

enslavement (meaning parasitism with the ciliate as parasite) than on true mutualistic relationships (see Nowack and Melkonian, 2010).

The incorporation of prokaryotes into symbiotic relationships is assumed to be a selective process, but some ciliates such as *Uronema* sp. are non-selective feeders (Alonso et al., 2000) and thus do not incorporate potential symbionts in a directed manner. Symbionts are well protected from the most important predators of pelagic bacteria when living inside ciliates which probably results in lower mortality rates than of free-living bacteria.

Endosymbioses most likely form in a "mature" stage of ecosystem development, e.g., when the limitation of food threatens reproduction and survival of potential partner organisms. High densities lead to increased intraspecific competition, which favors establishment of new consortia with highly efficient nutrient transfer among symbiotic partners (Nakajima et al., 2009). To facilitate such a transfer of nutrients, the host generates a symbiosome (i.e., a membrane surrounding the endosymbiont) that requires membrane modification to allow transport of otherwise excreted inorganic nutrients or metabolites to and from the symbionts (Yellowlees et al., 2008). The development of these transport systems suggests stable symbioses. On the other hand, it is likely that the host commonly replaces its endosymbionts due to Müller's ratchet—the genetic bottleneck effect that causes genetic depletion of the symbionts (Hackstein et al., 2004; Shinzato and Kamagata, 2010).

Interestingly, geographically separated populations of ciliates may be colonized by different symbiotic genotypes (Fokin et al., 2005; Summerer et al., 2008) and source communities. For example, Bernhard et al. (2000) investigated 15 ciliate species from the Santa Barbara Basin and only found two species without symbionts, ten species with ectosymbionts, five species with endosymbionts, and two species with both. Although the investigated environment is a highly selective one, and thus these findings cannot be easily generalized, it is worth underlining that about one third of the ciliate's body can be occupied by symbionts. This unambiguously demonstrates their potential importance for the host. Ciliates can host bacteria, archaea, and eukaryotes as symbionts. Most of the eukaryotic symbionts are photoautotrophs but there are also a few descriptions of other eukaryotes in ciliates available (e.g., Görtz and Dieckmann, 1987; Fokin et al., 2008). To date, 250 ciliate species are published harboring bacterial symbionts whereby symbiosis seems to be much more likely in nature than in laboratory cultures (see Fokin, 2012). To better understand such interactions, a deeper knowledge of the true diversity of ciliates carrying pro- and eukaryotic symbionts is required, particularly since the majority of aquatic ciliates may support symbiotic microorganisms (Fenchel et al., 1977; Finlay et al., 1996). In addition, the diversity of ciliates may be equalled or even exceeded by that of the symbionts as several ciliates simultaneously support two or more genotypes of symbionts (see Finlay et al., 1996).

TYPES OF CILIATE SYMBIOSES

To date, research has focused primarily on three major groups of symbionts in ciliates: phototrophic (including pseudo-symbiosis by chloroplast retention), chemosynthetic, and heterotrophic. Studied photoautotrophic groups include zoochlorellae in freshwater ciliates (e.g., *Stentor*, *Paramecium*), dinoflagellates and cryptophytes in marine ciliates (e.g., *Mesodinium*), and kleptochloroplasts in both marine and freshwater ecosystems (e.g., *Strombidium*). Lithoautotrophic symbionts studied include methanogens and sulfur oxidizers in anaerobic or microaerobic ciliates, and heterotrophic symbionts include *Paramecium* and its parasite *Holospora*, *Caedibacter* (the so-called "killer-symbiont"), and *Euplotes* with *Polynucleobacter necessarius*—a stable and inseparable symbiosis. These well-studied interactions appear to be highly specific and to result from long-term co-evolution, suggesting stable and highly conserved systems. Nevertheless, insights into interactions between the single symbiont species and different hosts, and comparison of sequence data from free-living with symbiotic microorganisms indicate that ciliate symbioses represent rather open and highly dynamic entities rather than closed systems (**Figure 1**).

PHOTOAUTOTROPHIC SYMBIONTS

Symbioses of ciliates with phototrophs have arisen independently and repeatedly in freshwater and marine systems (Esteban et al., 2010), resulting in a broad range of relationships. Most symbiotic phototrophs are eukaryotes or chloroplasts and it seems likely that the main function of eukaryotic endosymbionts is photosynthesis (Nowack and Melkonian, 2010). Interactions between the ciliate and its phototrophic symbionts range from brief associations of two facultative partners (e.g., limited retention of photosynthetic prey cells or their organelles) to permanent and even obligate symbioses (Nowack and Melkonian, 2010). Because the primary function of phototrophic symbionts is thought to be the supply of food to the host, such consortia are expected primarily in environments that are otherwise limited in organic food sources. However, mixotrophic ciliate species also commonly occur in eutrophic waters. They often exhibit phototactic behavior (Dolan, 1992), thereby providing their symbionts optimal conditions for photosynthesis. Nearly 25% of aquatic ciliates contain internal chloroplasts or algae (Foissner et al., 1999), making them less dependent on organic food supply from their environment. In addition, harboring a phototrophic endosymbiont may also allow the coexistence of competitors (Müller et al., 2012). These host-symbiont systems persist as obligate and facultative symbioses, whereby both often include a variable number of symbionts depending on season, light, temperature, and other environmental variables. Interestingly, the host seems to be capable of controlling the transfer of various compounds, in particular nitrogen, to the algal symbionts to regulate algal growth (Esteban et al., 2010). A complex cell-to-cell recognition process, in which modifications of algal cell wall structures are crucial, is potentially the basis for initiation and establishment of algal endosymbiosis (Lee et al., 1985).

In marine systems, the presence of kleptochloroplasts dominates besides a number of internal dinoflagellates and cryptophytes. In addition, *Strombidium purpureum* contains a non-sulfur purple bacterium capable of anoxygenic photosynthesis (Fenchel and Bernard, 1993a,b), and *Codonella* sp. is reported as the sole marine ciliate to harbor a cyanobacterium (Esteban

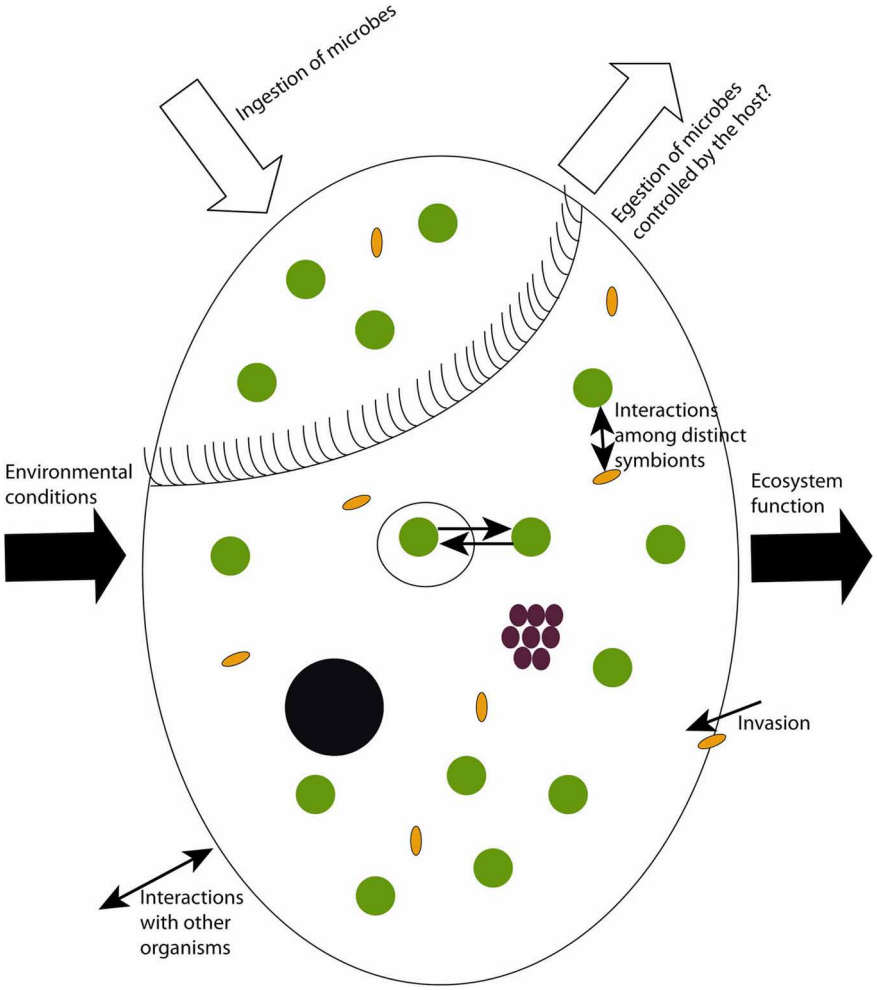

FIGURE 1 | Sketch of possible dynamics in symbioses with aquatic ciliates as host. Black circle = macronucleus, white big circle = food vacuoles, green circles = phototrophs, brown circles = chemoautotrophs, yellow ovals = heterotrophic prokaryotes.

et al., 2010). On the other hand, in freshwater systems so-called "zoochlorellae" (unicellular green algae living inside other organisms) predominate. So far, only a single freshwater ciliate species is known to contain the photoautotrophic phytoflagellate *Chlamydomonas* sp. as endosymbiont, whereas others also harbor kleptochloroplasts (Esteban et al., 2010). Sometimes, however, it remains unclear whether the internal compartments originate from endosymbiotic algae or chloroplasts.

Kleptochloroplasts

Kleptoplastidy is the ability of a heterotrophic organism to sequester plastids from algae by keeping the chloroplast intact. The plastids are maintained within the host and can be used for photosynthesis. Kleptoplastidy can therefore be defined as predation with farming of the prey organelles (Nowack and Melkonian, 2010). Kleptoplastids may represent an intermediate step in the acquisition of functional plastids or phototrophic symbionts (Nowack and Melkonian, 2010). For example, there are intact chloroplasts in the marine ciliates *Prorodon* and *Strombidium* that are not situated in vacuoles and are not digested by the host (Blackbourn et al., 1973). Dale and Dahl (1987) reported on the presence of symbiotic chloroplasts in *Strombidium*, *Prorodon,* and *Mesodinium rubrum*. Chloroplasts—often called "pseudo-symbionts"—also remain in *Tontonia* and *Laboea*, originating from incorporated prey (Dolan and Pérez, 2000). *Laboea strobila* sequesters photosynthetically functional chloroplasts derived from ingested algae (see Stoecker et al., 1988), and hence these ciliates require both light and algal food for its growth. In addition, the freshwater ciliate *Histiobalantium natans* contains sequestered chloroplasts and mitochondria which enable this aerobic ciliate to even survive and grow under anoxic conditions (Esteban et al., 2009). The duration of chloroplasts actively existing inside ciliates varies with species ranging from hours to a few days (Dolan, 1992) and environmental conditions, particularly light availability.

Mesodinium rubrum (= *Myrionecta rubra*) is a particularly interesting case. Although almost completely phototrophic, it is an obligate mixotrophic ciliate. Long included with zooplankton in plankton analyses (Crawford, 1989), it has a high photosynthetic activity. There is ongoing debate whether *M. rubrum*

contains chloroplasts or true symbionts, i.e., cryptomonades (see Esteban et al., 2010). What is clear is that they are retained throughout the year (Crawford, 1989) and need only less than one prey algal cell per generation for maximal growth (Hansen and Fenchel, 2006). It is possible that previous studies with *M. rubrum* containing symbionts or chloroplasts have been performed on functionally different clones or even different taxa (Montagnes et al., 2008). The great success of these symbiotic associations is based on the ciliate's motility that enables the otherwise passive planktonic autotrophic partner or chloroplast to optimize its light and nutrient conditions (Wilkerson and Grunseich, 1990).

Eukaryotic photoautotrophic endosymbionts

Strombidium and *Laboea strobila* often contain endosymbiotic algae as reported for the Baltic Sea (Mironova et al., 2009). This contradicts the observations that these ciliates primarily contain kleptochloroplasts. On the other hand, it seems likely that also here different functional strains were investigated. Another marine ciliate with phototrophic endosymbionts is *Maristentor dinoferus*, which harbors 500–800 symbiotic algal cells—all phylogenetically related to the dinoflagellate *Symbiodinium* (Lobban et al., 2002). Whereas the ciliates reshape at night, the symbionts are spread out in the cap during light, but are mostly moved into the stalk during darkness. These changes, however, are hardly a direct response to light availability (Lobban et al., 2002). Additionally, in *M. dinoferus*, mycosporine-like amino acids are most likely produced by the symbiont and protect the host against UV irradiation (Sommaruga et al., 2006). Therefore, a possible advantage from the endosymbiotic dinoflagellate is the protection of the host's intracellular macromolecules including DNA by shading the endosymbionts when ciliates are exposed to high solar UV irradiation—typically in transparent and oligotrophic waters (Sommaruga et al., 2006).

In freshwater systems, ciliates predominantly contain unicellular green algae, conventionally called zoochlorellae. We will keep this term in the following section. Reisser (1981) postulated for features of symbiotic associations with zoochlorellae: (1) a perialgal vacuole with one vacuole per alga which involves a recognition process between alga and host, (2) growth support of the host by symbiotic algae, and (3) host-symbiont specificity. Interestingly, algal symbionts of freshwater ciliates are of polyphyletic origin (Pröschold et al., 2011) indicating their independent development. Summerer et al. (2008) found a homogenous zoochlorellae group of different ciliate species from one lake, but clearly different zoochlorellae in one of those ciliate species occurring in another lake suggesting repeated incorporation of potential symbionts from the environment. All phototrophic endosymbionts in freshwater systems seem to support the host's growth by maltose excretion in a pH-depending manner (Reisser, 1981). One important factor controlling the endosymbiont's population size is the ciliate's relative need of the autotrophic mode of nutrition which is largely dependent on environmental conditions (Woelfl and Geller, 2002).

There are obligate as well as facultative symbioses with phototrophs: *Platyophrya chlorelligera*, for instance, contains zoochlorella cells at all life stages—even in cysts. On the other hand, euplotid ciliates seem to contain algae only in summer months (Dolan, 1992). To the contrary, for freshwater ciliates the absence or presence of zoochlorellae can be used for species identification as with *Stentor* sp. (Foissner and Wölfl, 1994) and seem thus to be permanent. *Paramecium bursaria* also harbors facultative algal symbionts, which show a membrane-membrane reaction in the host's food vacuole and, thus, prevent digestion (Dolan, 1992). Permanent symbioses in *Paramecium* seem to be restricted to *Chlorella* taxa (Summerer et al., 2007). These algae can provide up to 85% of the photosynthetic fixed carbon to the host (Muscatine et al., 1967), but large numbers of potential algal symbionts are digested (Karakashian and Karakashian, 1973). Non-digested algal endosymbionts are surrounded by a perialgal membrane produced from the host (Meier and Wiessner, 1989) and the endosymbionts cover between 10–56% of the ciliate's volume (Sud, 1968). In lab experiments, *P. bursaria* established stable symbioses with all tested *Chlorella* symbionts originating from various ciliates, but never with symbiotic *Chlorella* of the cnidarian *Hydra* sp. or free-living ones (Summerer et al., 2007). Despite clear preferences for their native *Chlorella*, the host-symbiont relationship in *P. bursaria* is flexible and adapts to environmental changes by accepting non-native *Chlorella* (Summerer et al., 2007). Algal symbionts of all *P. bursaria* strains of different origin could be assigned into one phylogenetic cluster apart from the other ciliate symbionts but split into two distinct lineages on the basis of biogeographic origin (Summerer et al., 2008). Species-specific symbioses were long assumed, but in different *P. bursaria* strains also different algal symbionts exist and at least five different algal species are known for this ciliate species (Pröschold et al., 2011). Summerer et al. (2008) stated that there is no more than one *Chlorella* genotype simultaneously in a single host population but they also cited studies on marine *Symbiodinium* symbioses which have clearly shown that one host can harbor more than one genotype at the same time (Santos et al., 2004). An interesting observation is that *Paramecium* with endosymbiotic zoochlorellae is not preyed as much as without algal symbionts (Berger, 1980). Another function is likely to be photoprotection of symbiotic *Chlorell*a by shading of sensitive cell compartments by a specific arrangement of the algal symbionts, depending on visible light and UV irradiation (Summerer et al., 2009). However, it may also be possible that there is higher grazing pressure on some ciliates containing algae or chloroplasts as they are more visible in the euphotic zone (Dolan, 1992) suggesting adaptations of the ciliates by either symbiosis or behavior.

The abundance of symbionts varies with species, size, and physiological status of the host, but there are at least hundreds of them (Foissner and Wölfl, 1994). As season also influences the host's physiology, it also affects the number of symbionts inside the ciliate host (Beaver and Crisman, 1989).

The ciliate *Climacostomum virens* contains *Chlorella* as symbionts in its cytoplasm and retains them over many generations through cell division and sexual production of the ciliate (Karajan et al., 2007). However, it is possible to grow ciliates as well as algae separately (Karajan et al., 2007). Another freshwater ciliate *Ophrydium naumanni* also contains *Chlorella* as observed in an oligotrophic South Andean lake in Argentina (Queimalinos et al., 1999). These symbionts give the ciliate the ability to effectively

exploit the water column in oligotrophic-high-light ecosystems (Queimalinos et al., 1999).

LITHOAUTOTROPHIC SYMBIONTS

Like phototrophic endosymbionts, lithoautotrophic symbionts can fix carbon and transfer some of their metabolites to the host. These symbionts are mainly found in anaerobic or microaerophilic ciliates. Endosymbiotic methanogens, for example, also occur in symbiotic ciliates of higher organisms and are thus responsible for the production of the green house gas methane, e.g., in cows. Symbiotic methanogenesis in aquatic systems, however, seems to be only of minor importance as phagotrophy under anoxic conditions only leads to slow growth rates (Fenchel and Finlay, 2010). Methanogenic symbionts exist in marine as well as freshwater ciliates and their methanogenic activity depends on the host's metabolism and growth yielding 0.35–7 pmol methane per ciliate and hour at maximal growth rates (Fenchel and Finlay, 1992). Methanogenesis does take place also at low oxygen concentrations in the environment, whereby the most characteristic interaction of methanogens and their host is the syntrophic transfer of hydrogen (Fenchel and Finlay, 2010).

Symbioses with methanogens have been formed repeatedly and independently (e.g., Shinzato and Kamagata, 2010), whereby distantly related ciliate species only contain closely related symbionts and closely related ciliate species only distantly related methanogens (Embley and Finlay, 1993). Despite multiple acquisitions of methanogenic endosymbionts, there is only one unique origin of hydrogenosomes, from mitochondria derived organelles producing hydrogen (Hackstein et al., 2004). There is a close phylogenetic relationship between the endosymbionts and free-living methanogenic archaea arguing for multiple acquisitions from environmental sources but also vertical transmission of endosymbionts has been reported (Van Hoek et al., 2000). The success of symbiosis reconstruction suggests that methanogenic symbiont and host ciliate might recognize each other probably due to membrane structures of the host but not by highly specific means, which could allow for a relatively easy symbiont replacement of anaerobic ciliates (Shinzato and Kamagata, 2010). Today, it is still unclear which factors are involved in the establishment and maintaining of symbiosis in anaerobic protozoa (Shinzato and Kamagata, 2010). It has been also suggested that there is no routine uptake of these endosymbionts but only if renewal of the symbiotic gene pool is needed (Hackstein et al., 2004), which is supported by the fact that strains of *Trimyema compressum* lost their methanogens when kept under cultural conditions (Goosen et al., 1990) without acquiring new ones. The host's benefit of harboring methanogens has been nicely demonstrated in cultures by inhibiting the methanogenic symbionts resulting in a reduction in the host's growth rate by 30% in two of three tested strains (Fenchel and Finlay, 1991a). This observation implies that different developmental stages of symbioses occur in different ciliate species.

Trimyema species do not require very specific methanogenic symbionts (Shinzato et al., 2007). The genus *Trimyema* in general contains different methanogenic species with also differences in stability of symbioses (Finlay et al., 1993). *Trimyema compressum* is the only ciliate of this genus known to harbor in addition to the methanogenic archaea some heterotrophic bacteria (Shinzato and Kamagata, 2010).

Narayanan et al. (2009) found *Methanosaeta*, an acetotrophic archaeum, in *Metopus* sp. The methanogenic endosymbionts of *Metopus paleformis* seem to have no effect on the methane concentration in the water (Biagini et al., 1998). On the other hand, *Methanoplanus endosymbiosus* is an endosymbiont in *Metopus contortus* with measurable *in situ* methanogenic activity and grows on hydrogen and carbon dioxide or formate (Van Bruggen et al., 1986). In this symbiosis, a constant number of 5000 hydrogenosomes and 3500 methanogenic symbionts occur, whereby the reproduction of methanogens controls the growth cycle of the host (Fenchel and Finlay, 1991b). *Plagiopyla nasuta* contains the methanogenic *Methanobacterium formicium* which could not be isolated so far (Goosen et al., 1988) and, hence, its specific role for the host is unknown.

The sulfur-oxidizing symbionts, instead, may function to link the sulfur cycle with cycling of carbon and nitrogen (Edgcomb et al., 2011). The host can serve as a shuttle between oxic and anoxic water layers so that the symbionts can use H_2S or methane as an energy source and oxygen as an electron acceptor whereby also nitrate can be used at least periodically (Cavanaugh et al., 2006). The symbionts have the same generation times as the host and can reach high densities on it (Fenchel and Ramsing, 1992).

In *Zoothamnium niveum* the sulfur-oxidizing bacterium Candidatus *Thiobios zoothamnicoli* was found (Dubilier et al., 2008). Candidatus *T. zoothamnicoli* is only distantly related to previously identified groups of thiotrophic symbionts with highest similarity to a free-living strain (Rinke et al., 2006). Ectosymbionts form a monolayer on *Z. niveum*, which covers the entire colony except for parts of the stalk (Polz et al., 2000). Once in a while, the ciliates rapidly contract and completely immerse themselves in the sulfidic boundary layer. During the subsequent slow expansion they drag sulfidic water into the oxic ambient water (Ott et al., 1998). *Zoothamnium* feeds largely on the symbiotic bacteria detached after contracting (Ott and Bright, 2004) and represents the major food source for the ciliate (Polz et al., 2000). *Zoothamnium niveum* was also found to be almost completely covered by chemolithoautotrophic sulfur-oxidizing bacteria in a mangrove island of the Belize Barrier Reef, which give them a white color (Ott and Bright, 2004).

Only half of the biomass of the mouthless marine interstitial ciliate *Kentrophorus* is indeed ciliate, the remainder is a coat of sulfur-oxidizing bacteria, which is the basis of the ciliate's diet by periodically pushing in its cell surface and digesting the bacteria (Fenchel and Finlay, 1989). These bacteria need both sulfide and oxygen for autotrophic carbon fixation which only coexists in nature in narrow, changing and often unpredictable microzones (Ott and Bright, 2004).

ORGANOHETEROTROPHIC SYMBIONTS

The above mentioned interactions represent mutualistic relationships or interactions which are at least positive for the host whereas heterotrophic symbionts also include transitions to parasites. In general, there might be larger difficulties of integrating a prokaryotic cell into a eukaryotic system than vice versa,

mainly due to differences in genome structures and the need of developing a protein transport mechanism to the prokaryotic symbiont (Nowack and Melkonian, 2010). Ciliates may represent genetic "melting pot" promoting cross-species genetic exchange as a result of the co-occurrence of different intracellular bacteria (Nowack and Melkonian, 2010; Lamrabet et al., 2012).

Symbionts providing defense for the host have only been identified for ciliates so far (Gast et al., 2009). Fokin et al. (2005) reported that there are about 200 ciliate species recorded with bacterial symbionts, which is likely to be only a small part of their true number. Most of the marine ciliates investigated by TEM harbor a flora of ecto- and endosymbiotic bacteria (Fenchel et al., 1977). Finlay and Esteban (1998) also stated that most ciliates host endo- or ectosymbiotic organisms, however, they also reported that the diversity and ecological role of these symbionts are hardly investigated. Some of these intracellular bacteria are surrounded by the host's membrane; others not (Görtz and Fokin, 2009). Unfortunately, for the majority of the endosymbiotic bacteria reported in protists, not much more than a morphological description is available, precluding any conclusions about their physiological role as well as a clear recognition of the bacteria as endosymbionts, pathogens, or prey (Nowack and Melkonian, 2010). Intracellular microorganisms typically show low abundance in the host cell (Görtz and Brigge, 1998) and can be found in various cell compartments and in different ciliate species (Görtz, 2001). The symbioses with heterotrophic bacteria appear as highly variable and dynamic with differences on ciliate population levels—and asides the three above mentioned model systems with (1) *Holospora*, (2) *Caedibacter,* and (3) *Polynucleobacter* these interactions are poorly understood and studied.

Ciliate symbioses with Holospora spp.

The first description of *Holospora* bacteria was already given in 1890 by Hafkine (Fokin et al., 1996). The endonuclear symbiont *Holospora obtusa* is the closest relative to mitochondria known to date (Görtz and Fokin, 2009). There are at least two groups of *Holospora* in *Paramecium* which differ in behavior during host division (Fokin et al., 1996). *Holospora* may be regarded as truly parasitic but has no effect on the host's growth rate under favorable conditions (Görtz and Brigge, 1998). Their metabolic interactions leading to a higher temperature tolerance of *Paramecium caudatum* infected with *Holospora obtusa* is not understood at all (Dohra et al., 1998). Modern functional genomics and proteomics could potentially resolve the ecological role of such interactions.

Ciliate symbioses with Caedibacter spp.

The transition from mutualism to parasitism can be highly variable as for the killer-symbiont *Caedibacter* spp., which is able to produce a toxin against potential competitors but may also overgrow the host cell (Schmidt et al., 1987). So far, neither the killing toxins nor the mechanism by which paramecia infected with *Caedibacter* resist being killed have been identified (Görtz and Brigge, 1998). It is known, however, that only *Caedibacter* bearing phages or plasmids may confer the killer trait to their host besides in *C. taeniospiralis* a plasmid is responsible for the formation of R-bodies and thus toxin production (Quackenbush and Burbach, 1983; Heruth et al., 1994). Interestingly, when a symbiont-free host cell, which previously harbored killer symbionts, is infected with nonkiller-symbionts, the ciliate proves to be an active killer again. This suggests that extrachromosomal elements (e.g., plasmid) or phages from the killer symbiont are left in the ciliate and can be introduced into the nonkiller bacterium, which in turn is transformed into a killer symbiont (Görtz and Brigge, 1998). Killer symbionts are also found in other ciliates such as *Euplotes*, *Paurauronema acutum* (see Görtz and Brigge, 1998), and *Spirostomum ambiguum* (Fokin et al., 2003).

Ciliate symbioses with Polynucleobacter necessarius

Polynucleobacter necessarius are considered as the most recent known obligate symbionts at the moment (Vannini et al., 2007). The ciliate *Euplotes* needs *Polynucleobacter* for its survival (Heckmann, 1975; Heckmann et al., 1983; Vannini et al., 2005a), whereby *Polynucleobacter* is neither infectious nor pathogenic. Instead, the bacterium works more like an organelle, but its precise function inside the ciliate is still unknown. It has been postulated that it descends from an early symbiont that compensated for a metabolic deficiency of the host (Heckmann et al., 1983) suggesting a stable established symbiosis with a defined physiological function. Recent investigations show that *Polynucleobacter* interferes with the glycogen metabolism of their hosts (Vannini et al., 2007). *Polynucleobacter* is also found in the brackish ciliate *Euplotes harpa* where its removal stops the reproductive cycle of the ciliate (Vannini et al., 2005a). In two of three tested strains of *Euplotes harpa*, *Polynucleobacter* co-occurred with other symbiotic bacteria, whereby the other bacteria showed only low abundances (Vannini et al., 2005a).

Ciliate symbioses with other heterotrophic bacteria

The ecological consequences of bacterial symbioses in protozoa are manifold and can even constitute a potential risk for human health when comprising potential pathogenic bacteria (Görtz and Michel, 2003; Ferrantini et al., 2007). Most of the symbionts are not infectious, but the uptake of symbionts can result in a co-infection with infectious microbes (Fokin et al., 2004). Relationships between protists and pathogenic or pathogen-related bacteria including *Legionella*, *Chlamydia*, and *Rickettsiaceae* indicate that there may be potential risks. Protists from sewage plants and composters are frequently infected with microorganisms (Görtz and Maier, 1991; Görtz and Brigge, 1998), e.g., *Rickettsia*-like organisms. However, in the cytoplasm of a marine *Diophrys* species *Rickettsia*-like organisms were also present (Vannini et al., 2003). To be more particular, the ciliate *Diophrys appendiculata* from the Baltic Sea contains specific *Rickettsiaceae*, which possess an independent phylogenetic position within this group (Vannini et al., 2005b). *Euplotes harpa* even contains two *Rickettsia*-like organisms: Candidatus *Anadelfobacter veles* and Candidatus *Cyrtobacter comes* (Vannini et al., 2010). These ciliates represent suitable model systems to study interactions between potentially pathogenic bacteria and their eukaryotic host as well as the resulting ecological consequences (Vannini et al., 2005b). For example, Ogata et al. (2006) postulated that amoeba-like ancestral protists may have served as a genetic "melting pot" where the ancestors of *Rickettsiaceae*

and other bacteria promiscuously exchanged genes, eventually leading to their adaptation to the intracellular lifestyle within eukaryotic cells.

Another example for pathogenesis via aquatic ciliates is the fish parasite *Ichthyophthirius multifiliis* containing the following endosymbiotic bacteria: an alphaproteobacterium related to *Rickettsia*, *Sphingobacteria,* and *Flavobacterium columnare*—all with unknown function (Sun et al., 2009). As not all ciliates of this species contain detectable endosymbionts, it is unlikely that endosymbionts play a critical role for the host's physiology. Further, it is unknown whether they play a role for the pathogenic infections by the ciliate or whether they directly affect the immune response of infected fish (Sun et al., 2009). *Francisella noatunensis* is another endosymbiont with potential pathogenic capabilities, which occurs naked and without any other symbiotic genotypes in the marine ciliate *Euplotes raikovi* (Schrallhammer et al., 2011). *Francisella* is a facultative intracellular bacterium, causing severe disease in a broad range of animals including fish. Consequently, aquatic ciliates can serve as reservoirs for pathogenic bacteria with potential severe consequences for animal and human health (Schrallhammer et al., 2011).

The symbiotic relationship between the ciliate *Euplotes magnicirratus* and the bacterium Candidatus *Devosia euplotis* is an example for a permanent and species-specific relationship (Vannini et al., 2004), whereby the bacterium supports the digestion of food organisms (true dependence upon a symbiont, Vannini et al., 2003). Aside of the "classical endosymbiosis," *Euplotidium* have *Verrucomicrobia*-like ectosymbionts (= **epixenosomes**), which protect their host against the predator *Litonotus lamella* by committing suicide (Rosati et al., 1999). These ciliates keep at least some of their ectosymbionts also in culture (Petroni et al., 2000).

Another type of symbiotic relationships between ciliates and bacteria are rod-shaped **xenosomes**, found in *Paurouronema acutum*, which are Gram-negative bacteria comparable in size to *Rickettsia*-like organisms. They exclusively occur in the host cytoplasm and divert together with their host (Soldo, 1987). These xenosomes have multicopies of their genomes (Soldo, 1986). *Paurouronema* is infected by direct penetration of the symbiont through the ciliate's cell membranes, and thereafter only a single xenosome is required to establish a permanent infection (Soldo et al., 1993).

Strains of *Trimyema compressum* contain in addition to methanogens also a non-methanogenic prokaryote which can be lost under laboratory culture conditions (Goosen et al., 1990). This suggests that symbioses in *Trimyema* strains are not obligatory and may have a transient character. One of the *Trimyema* bacterial symbionts is only distantly related to other known bacterial species (85% and less) belonging to the *Syntrophomonadaceae* (*Firmicutes*). This suggests that this symbiont is specifically associated to strains of *Trimyema* (Shinzato et al., 2007). Although the absence of the bacterial symbiont after antibiotic treatment considerably decreased the host's growth, the precise role of the bacteria for the ciliate is still unknown (Shinzato et al., 2007). However, it has been supposed that differences in the host's behavior among various strains of *Trimyema* sp. are due to different endosymbiotic communities (Goosen et al., 1990). The presence of multiple symbionts yields a more complex picture of potential symbiotic functions and may even result in a complex functional cycle. For example, in a ciliate closely related to *Parduzcia orbis*, three different types of endosymbionts are present. They are organized within membrane-bound sub-cellular regions and comprise one or two sulfate reducers, a methanogen, and a Type I methanotroph forming synergistic metabolism (Edgcomb et al., 2011).

Species-specificity of symbiotic interactions

Based on morphology each ciliate species harbors its specific microbial flora (Fenchel et al., 1977). The marine sediment ciliate *Geleia fossata* can host among 2000–10,000 epibiotic cells. In a few isolates of *Stentor* and *Spirostomum* a number of so far unknown bacterial species have been observed (Görtz, 2001). For *Paramecium* more than 60 intracellular distinct bacteria have been described (Görtz and Fokin, 2009), unfortunately often without depositing the 16S rRNA gene sequence information in public databases. The vent ciliates of the *Folliculinopsis* group harbor multiple phylogenetically distinct symbionts located in different parts of the cell (Kouris, 2009). Symbionts of the ciliate genus *Spirostomum* can be located in the cytoplasm, mitochondria, and macronucleus (Fokin et al., 2003). In general, symbionts in mitochondria are rare and only a minor part of the population is infected as demonstrated for *Halteria geleiana* (Yamataka and Hayashi, 1970) and *Urotricha ovata* (De Puytorac and Grain, 1972). Both *Spirostomum* species investigated seem to permanently maintain their endosymbionts and were—at least partly—colonized by different bacteria (Fokin et al., 2005). The ciliate *Parablepharisma* even shows a specific adaptation for hosting ectosymbionts (Fenchel et al., 1977) leading to a highly host-specific symbiotic bacteria-host relationship. Moreover, the ciliate *Frontonia leucas* hosts an alphaproteobacterial symbiont in its macronucleus (Fokin and Schweikert, 2003). The unequal distribution of two different bacteria in the cytoplasm of *Paramecium* suggests that conditions in various parts of the cytoplasm and other parts of the host cell favor for distinct bacteria and their maintenance in each compartment of the host cell (Fokin et al., 2000). It is likely that the host leads the symbiont to the "right" place with its cytoskeleton (Fokin et al., 2000), which indicate highly specific interactions between the bacterial symbiont and the ciliate host. Different features of the bacteria in regard to their infectivity and their residence place in the host cell can be regarded as a further indication for a highly specific mode of interaction and a great variety of intracellular bacteria (Fokin et al., 2000).

CURRENT AND FUTURE APPROACHES TO THE STUDY OF CILIATE SYMBIOSIS

Studies on symbiotic interactions traditionally include microscopic investigation and phylogenetic characterization of the symbionts using DNA-based approaches. While microscopy (e.g., light microscopy, electron microscopy, confocal laser scanning microscopy) and phylogenetic analysis (e.g., 16S rRNA gene sequencing) allow very detailed structural analysis of host-symbiont interactions they are limited in the study of physiological and functional aspects. As the majority of symbionts do not grow in pure culture most studies today rely on indirect

approaches to investigate symbiosis in ciliates (Cavanaugh et al., 2006). Below we provide an overview of current and future technologies that we consider useful in the study of ciliate symbioses.

Symbiotic bacteria can be studied at several levels: morphological (ultra structural), physiological, biochemical as well as on a molecular level (Fokin et al., 2003). The rapid development of new molecular tools greatly improved our understanding of biological mechanisms including organismic interactions (Kitano, 2002; Medina and Sachs, 2010; Hongoh, 2011; Weckwerth, 2011). One major driver of these developments is sequencing technology, which is often key to various studies. Today, next-generation sequencing (NGS) technologies allow high-resolution analysis of single organisms or complex communities on a molecular level at very low costs (Metzker, 2010). More recent developments also put RNA sequencing (transcriptomics) or proteome analysis (proteomics) into focus. The study of mRNA and/or proteins has the advantage that it reveals not only structural information but also indicates genes and metabolic pathways actively expressed by the organisms studied under the conditions the sample was taken (Schneider and Riedel, 2010; Toledo-Arana and Solano, 2010). Using genomics, transcriptomics, and proteomics separately or in combination we can now approach a variety of research questions including: simple phylogenetic profiling of symbiotic communities; comparative genomics of symbiotic microorganisms or hosts; population dynamics of hosts and/or symbionts; or host-symbiont interactions on a molecular level (Kleiner et al., 2012).

While the methods themselves are often routine, sampling and sample preparation became more and more challenging. In particular, working with symbionts can be very difficult due to the close spatial and functional association between hosts and symbionts. For example, ciliates do not only harbor symbiotic bacteria but also graze on them. But how to distinguish between symbiotic bacteria and those contained in food vacuoles? A physical separation of the different fractions is therefore essential—especially when using nucleic acid based approaches (e.g., for symbiotic community profiling) to minimize false interpretations of the data. One option is to label dead cells with propidium monoazide ("live-dead staining") followed by flow cytometry to separate dead bacteria from living symbionts (Nocker et al., 2007). More recently single-cell approaches became very popular allowing molecular characterization (e.g., genome sequencing) of single bacterial cells (Woyke et al., 2010). For such studies the single cells are usually separated by: (1) micromanipulation using a microscope equipped with a proper micromanipulation device, (2) flow cytometry, or (3) microfluidics (e.g., Hong and Quake, 2003; Brehm-Stecher and Johnson, 2004). Furthermore, density gradient centrifugation can be used to separate bacterial cells from tissues or other organisms (Woyke et al., 2006). Micromanipulation has been successfully used to isolate endosymbiotic bacteria from protists that live in the termite hindgut (Hongoh et al., 2008). The isolated endosymbionts were further characterized by whole genome sequencing revealing that they can fix nitrogen supplying the host with essential nitrogenous compounds.

Working with only a few cells or single cells holds another challenge that needs to be considered: limited nucleic acids and protein concentrations. While culture organisms can be grown to a certain density this is not applicable for single cell approaches. For studies only involving DNA, multiple displacement amplification (MDA) can be used to generate enough material from a few fg of template DNA for subsequent studies (Lasken, 2006). However, there are no standard protocols available yet if working with very little amounts of RNA or proteins for respective transcriptomics or proteomics studies.

Alternatively to working with single-cells, it is also possible to use meta-*omics* approaches (e.g., metagenomics, metatranscriptomics, metaproteomics) to explore host-symbiont interactions (Kleiner et al., 2012). Metagenomics sequencing of bacterial community is a routine application nowadays and can be performed without many difficulties. But it can become problematic when eukaryotic genomes are contained in the samples. While bacterial genomes have sizes ranging from 160 Kbp to 10 Mbp a eukaryotic genome can be as large as 670 Gbp (McGrath and Katz, 2004). So performing metagenome sequencing on a mixture of pro- and eukaryotes can be very challenging and most likely biased toward the eukaryotic host. However, if eukaryotes can be excluded (e.g., by removing the macronucleus of the ciliate host) metagenomics can be a very powerful approach, especially when working with unknown communities. For metatranscriptomics studies, the prokaryote-eukaryote ratio is not as eminent since eukaryotic mRNA has a polyA-tail and thus can be easily separated from the bacterial fraction.

Besides the described sequencing-based approaches there are other methods available that can be used to study organismic interactions. One of these alternatives is microarrays as shown by Barnett et al. (2004). Here a DNA microarray was developed containing probes for the host as well as the symbiont on a single chip allowing to investigating gene expression in both partners simultaneously. However, such microarray studies are only possible if the underlying genomics information of the symbiotic partners is available to design the oligonucleotide probes for the chip. Therefore, microarrays are only of limited use if symbiotic communities are unknown. Genomic or gene sequence information can be also used to design oligonucleotide probes for fluorescence *in situ* hybridization (FISH). In microbial ecology FISH is traditionally being used to label microbes with oligonucleotide probes against the small-subunit ribosomal RNA to distinguish between different phylogenetic lineages (Amann et al., 2001). More recent studies also used FISH to target functional genes by a so-called recognition of individual genes FISH (RING-FISH) (Zwirglmaier et al., 2004; Dziallas et al., 2011). Either approach will be of great use for future studies on symbiotic interactions since they allow to determining how symbionts are structurally and morphologically associated with their hosts and which functions they are able to carry out.

Structural and functional characterization of host-symbiont interactions can be further complemented by additionally collecting metabolic information of the symbiosis. This allows linking genomic and transcriptomic information to real metabolic activities. Metabolomics is a relatively new research field aiming to identify metabolites or intermediates of cellular processes (Macel

et al., 2010). The direct study of metabolites has the advantage that it really shows what pathways are active in a cell or community, which cannot be determined by only transcriptomic or proteomic analyses. Another interesting technology in this respect is high-resolution secondary ion mass spectrometry (NanoSIMS) that enables the study of microbial physiology and the use of certain elements (e.g., N, C, S) on a cellular level (e.g., Behrens et al., 2012).

Genomic tools and data available (large-scale sequencing, published genomes, and bioinformatics) could provide increased resolution in the study of bacterial diversity. Standard methods of determining taxon composition using16S rRNA OTUs are known to provide only "coarse" estimates of functional diversity or evolutionarily distinct populations. Improved methods of "species" delineation in bacteria that use DNA sequences will be provided by alternative gene regions (e.g., pseudogenes, intergenic regions; Gomez-Valero et al., 2007) and coalescent-based methods (e.g., Barraclough et al., 2009). Jousselin et al. (2009) used the approach with intergenic (neutrally evolving) regions to delineate fine-scale bacterial "species" and observed a previously unknown host-symbiont co-speciation. Furthermore, genomic information can be also used to develop novel isolation strategies for yet uncultured symbiotic microorganisms as shown previously (Tyson et al., 2005). That would provide the chance of studying "symbioses in action" including the establishment of symbioses of isolates and symbiont-free ciliate hosts.

All these new methods have the potential to provide more detailed information on symbioses and their impact on the respective ecosystem. For example, NanoSIMS may enlighten material flow between different symbionts or symbionts and host and thus give further information on the complex functional interaction within one host and also the dependence of the consortium on different compounds from the environment. Thus, these cutting-edge technologies will not only enlarge our databases, but will help to solve many unanswered ecological questions and also to discover new problems.

EMERGING QUESTIONS

As is evident from our review, there are many open questions regarding the origin, evolution, maintenance, ecology, and biogeochemical implications of ciliate symbioses. Many of these questions are quite general and their study would provide a better understanding of aquatic ecology, biogeochemistry, and evolution more generally. While several new methods (above) hold promise, we feel that new conceptual approaches are needed to unravel the hidden secrets of bacteria-ciliate interactions. We highlight a number of topics below.

DO ENVIRONMENTAL CONDITIONS DETERMINE THE NATURE OF SYMBIOSES?

The independent and repeated establishment of different symbioses between aquatic ciliates and microorganisms suggests that environmental conditions at least partly regulate incorporation, maintenance, and termination of these symbioses. Unfortunately, the function of many symbionts is unknown and hence it is still unclear whether there is a directed establishment of symbioses for required functional traits. For example, it seems likely that many aquatic ciliates incorporate and maintain nitrogen-fixing bacteria under nitrogen-limited conditions. If proven to be correct, this would also imply a highly dynamic establishment of symbioses with only loosely linked microbial partners. Alternatively, potentially beneficial symbionts may be retained in the ciliate in low numbers and with reduced activity also when not needed. This might be of particular advantage in unstable and fluctuating environments.

WHAT ARE THE EVOLUTIONARY CONSTRAINTS ON SYMBIOSIS?

Our review shows that ciliate symbiosis has arisen across a broad range of taxa and environments; nonetheless, little is known about the evolutionary basis of the symbiosis. One important question is whether symbionts are species-specific or can affect a range of hosts? If species-specific symbiosis is common, then it may be that host-symbiont co-speciation has given rise to ciliate diversity. If a few small lineages form most symbioses, it suggests only one or a few genomic or genetic mechanisms may be involved, whereas if many unrelated lineages can form symbioses there may be functional redundancy. These and similar questions require broad taxonomic sampling that is increasingly available with large-scale sequencing methods (above) and can employ sequence-based delineation methods (e.g., Jousselin et al., 2009).

WHAT ARE THE ECOLOGICAL FUNCTIONS OF SYMBIONTS?

Conventionally, it is thought that stable symbioses are mutualistic relationships in most cases. On the other hand, it is likely that a number of symbioses are established just by chance, e.g., by co-ingestion of infectious symbionts. However, functions of microbial symbionts not only affect the host but can have great implications for global matter cycling, as by nitrogen fixation, phosphorus storage or sulfur transformation, or the ecosystem, as by toxin production, oxygen production in anoxic environments or altered infectivity of pathogenic hosts.

Related to the functionality of symbioses it remains unclear which precise mechanisms and interactions between hosts and symbionts lead to stable and obligate symbioses? Additionally, can neutral symbioses without any measurable effect on both partners be stable? And when symbiosis can be also seen as a trap for both partners: can this symbiosis be stable and how do the partners interact as consortium with their environment including uninfected host organisms?

ARE CLOSED MATTER CYCLES FORMED?

Edgcomb et al. (2011) report on the existence of methanogens and a methanotroph in an individual ciliate suggesting the possibility of closely linked biogeochemical cycles, but even more complex cycles such as for nitrogen seem to be possible. Such complex biogeochemical cycles require multiple symbioses consisting of more than two partners and thus highly specific adaptations and interactions (possibly multitrophic) are necessary. A complex network of symbiotic partners in a single ciliate host is supported by the observation that often more than one symbiont species can be found in a single ciliate.

WHICH SPECIFIC MECHANISMS ALLOW THE HOST TO CONTROL THE SYMBIONT'S PHYSIOLOGY?

This question includes not only the need of control mechanisms for metabolic exchange (e.g., nutrient transfer), but also of communication (i.e., signaling). How does the host recognize which symbiont is beneficial at a given time? How can it allocate nutrients to, e.g., a phototrophic symbiont, but not to a symbiont promoting digestion of the host's food? Which mechanisms are involved in the host-symbiont communication?

ARE THERE DIFFERENT TYPES AND MECHANISMS FOR SYMBIOSES IN MARINE VERSUS FRESHWATER HABITATS?

Some closely related ciliates occur in marine, brackish, and freshwaters. Thus, for these species different mechanisms in the establishment and maintenance of symbioses are unlikely. However, marine and freshwater ecosystems differ besides salinity in many parameters such as limiting nutrients. This may result in the acquisition of specific microbial functions. For example, most marine systems are limited by nitrogen compounds, thus, acquiring bacteria with the capability of fixing nitrogen is highly advantageous. For these bacteria the host must provide an anoxic or microoxic environment to fix gaseous nitrogen. In freshwater systems on the other hand, phosphate is mainly the limiting factor which may result in symbionts capable of storing phosphate under favorable conditions. Thus, the host might need to move to zones rich in phosphate and establish behavior to circulate this phosphate rich water through its cell.

ARE THERE DIFFERENT FORMS OF SYMBIOSES IN ANAEROBIC AND AEROBIC HABITATS?

If yes, what can we learn for biogeochemical cycling and evolution (e.g., adaptation and speciation of the ciliate and the microbial symbionts)? Containing methanogens seems to be restricted to ciliates living in anoxic or microoxic environments. This symbiosis includes tight coupling between the host's hydrogenosomes and the symbionts, thus even the evolution of hydrogenosomes may represent adaptation to symbiotic lifestyle and a unique niche for symbionts. Another special environment is the bridge between oxic and anoxic conditions where ciliates may favor their symbionts' growth by actively moving between these two zones. On the other hand, ciliates in the oxic zone may provide niches for anaerobic or microaerophilic bacteria in some of their cell compartments.

DO SYMBIOSES INCREASE THE SPEED OF MICROBIAL GENE EXCHANGE?

Hereby, it is also interesting if this genetic exchange happens predominately between symbiont and host, symbiont and symbiont or even between symbiont and prey. Additionally the exchange and change of the symbiont's genomic signature after genetic depletion is highly interesting. Which evolutionary principles can be dissected from microbe-ciliate symbioses (e.g., the role of horizontal gene transfer or virus and phages as genetic vectors)?

CONCLUSIONS

Many ciliates contain microorganisms such as algae, Bacteria, and Archaea (**Table 3**, **Figure 2**) and interact with these in manydifferent and often unknown ways. As internal algae are easy to recognize using a microscope most studies published to date have been focused on these symbionts—also due to the fact that their interactions with ciliates are mutualistic. Nonetheless, internal bacteria can also comprise commensalistic and parasitic symbionts which might have a great effect on the ecosystem by influencing their host.

Ecosystem functioning is an important and often little understood parameter in biodiversity research. Unfortunately, symbionts are hardly taken into account although their capability of photosynthesis, sulfur transformation, methanogenesis and

Table 3 | Overview of ciliates with symbionts.

	Examples for ciliate genera	
	Marine, brackish	**Freshwater**
PHOTOAUTOTROPHIC SYMBIONTS		
Endosymbionts	*Codonella*	Climacostomum
	Euplotes	*Disematostoma*
	Laboea	*Euplotes*
	Maristentor Mesodinium	*Frontonia*
	Platyophrya	*Ophrydium*
	Strombidium	*Paramecium*
		Stentor
		Tetrahymena
Chloroplasts	*Laboea*	*Histiobalantium*
	Mesodinium	*Perispira*
	Prorodon	
	Strombidium	
LITHOAUTOTROPHIC SYMBIONTS		
Endosymbiotic	*Caenomorpha*	*Caenomorpha*
methanogens	*Metopus*	*Metopus*
	Parduzcia	*Trimyema*
	Plagiopyla	
	Trimyema	
Ectosymbiotic	*Kentrophorus*	
sulfide oxidizers	*Zoothamnium*	
ORGANOHETEROTROPHIC SYMBIONTS		
Mutualistic	*Diophrys*	*Euplotes*
endosymbionts	*Euplotes*	*Paramecium*
	Parauronema	
	Parduzcia	
Mutualistic	*Euplotidium*	
ectosymbionts		
Parasitic		*Paramecium*
endosymbionts		*Stentor*
Endosymbionts with	*Euplotes*	*Frontonia*
unknown effects	*Folliculinopsis*	*Halteria*
	Parauronema	*Ichthyophthirius*
	Spirostomum	*Paramecium*
		Spirostomum
		Stentor
		Urotricha
Ectosymbionts with	*Geleia*	*Loxophyllum*
unknown effects	*Paraspathidium*	
	Tracheloraphis	

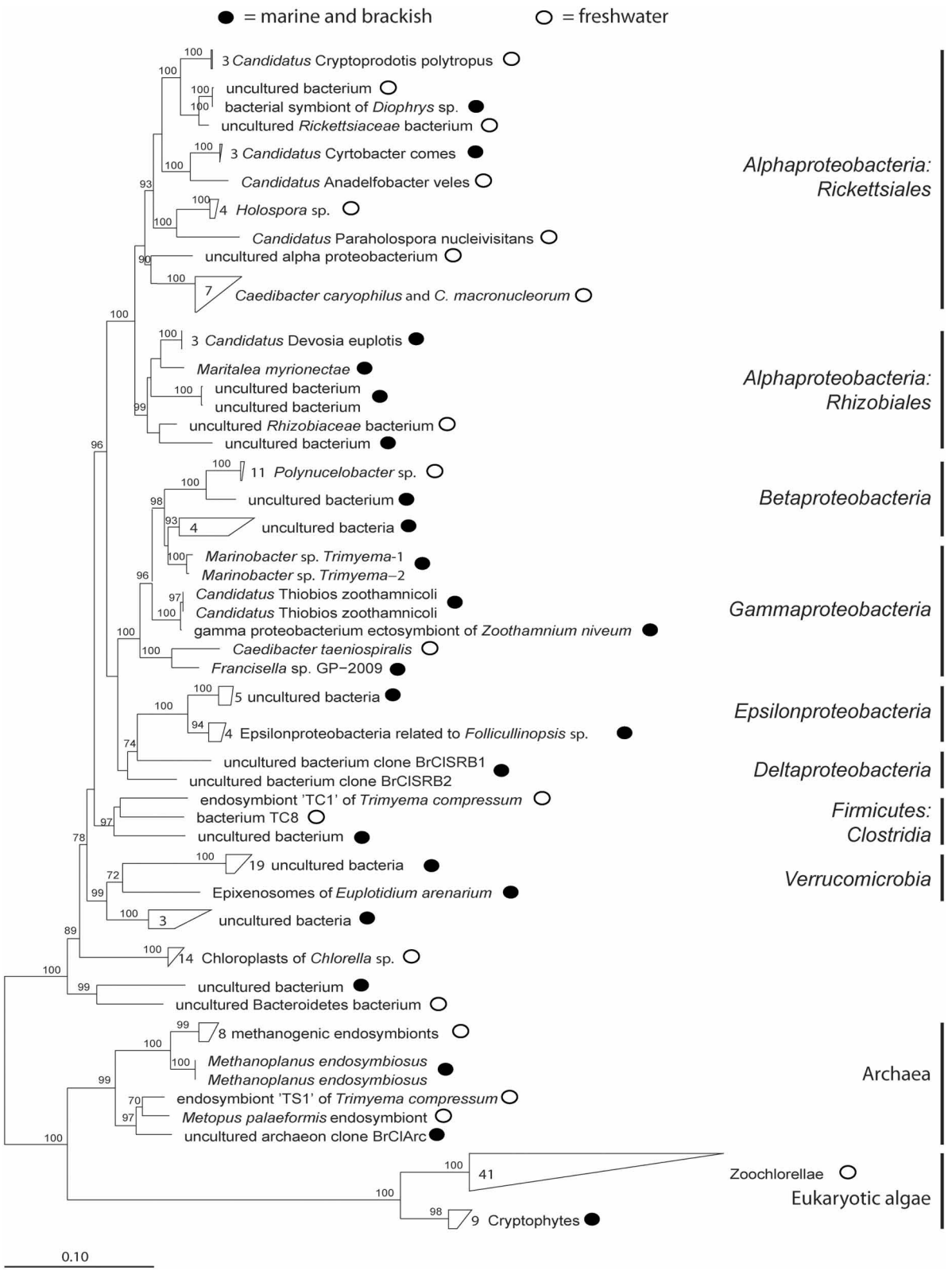

FIGURE 2 | Phylogenetic tree of published symbionts in aquatic ciliates (accession numbers are given in Table A1). The tree was calculated with FastTree using the aligned sequences from ARB-SILVA. Bootstrap values are only given ≥70.

their demand on different elements including iron and magnesium is well known. To calculate their importance for regional and global matter cycling will be an interesting and challenging task in upcoming research by also determining their impact for other element cycling such as nitrogen and phosphorus.

Our review shows how diverse and complex symbioses between aquatic ciliates and associated microbes can be and how many gaps in our knowledge still exist. In particular, gaining more information on how symbioses are established and maintained not only extends our scientific knowledge but also may give new insights into species evolution and material cycling and may underline that teamwork can outcompete the individual.

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APPENDIX

Table A1 | Used published sequences (order according to the phylogenetic tree in Figure 2).

Sequence names	Accession numbers
3 *Candidatus* Cryptoprodotis polytropus	FM201293-5
Uncultured bacterium	AF523878
Bacterial symbiont of *Diophrys* sp.	AJ630204
Uncultured *Rickettsiaceae* bacterium	GQ870455
3 *Candidatus* Cyrtobacter comes	FN552696-8
Candidatus Anadelfobacter veles	FN552695
4 *Holospora* sp.	JF713682-3, X58198, AB297813
Candidatus Paraholospora nucleivisitans	EU652696
Uncultured alpha proteobacterium	FM201297
7 *Caedibacter caryophilus* and *C. macronucleorum*	AM236090-3, X71837, AJ238683, AY753195
3 *Candidatus* Devosia euplotis	AJ548823-5
Maritalea myrionectae	EF988631
Uncultured bacterium	FN999956, FN999980
Uncultured *Rhizobiaceae* bacterium	FM201296
Uncultured bacterium	FN999955
11 *Polynucleobacter* sp.	AJ585515-6, AJ811013-4, AM398080-1, AM397067, CP001010, AM398078, X93019
Uncultured bacterium	FN999982
4 uncultured bacteria	FN999996, FN999962-4
Marinobacter sp. *Trimyema*-1, 2	AJ292527, AJ292528
Candidatus Thiobios zoothamnicoli	AJ879933, EU439003
Gamma proteobacterium ectosymbiont of *Zoothamnium niveum*	AB544415
Caedibacter taeniospiralis	AY102612
Francisella sp. GP-2009	FN398155
5 uncultured bacteria	FN999957-60, FN999981
4 Epsilonproteobacteria related to *Follicullinopsis* sp.	GU253370-3
Uncultured bacterium clone BrCISRB1, 2	JF327425, JF327424
Endosymbiont 'TC1' of *Trimyema compressum*	AB118592
Bacterium TC8	AB118593
Uncultured bacterium	FN999965
19 uncultured bacteria	FN999947-54, 69-79
Epixenosomes of *Euplotidium arenarium*	Y19169
3 uncultured bacteria	FN999966-67, 46
14 Chloroplasts of *Chlorella* sp.	EF030588-99, 602-3
Uncultured bacterium	FN999968
Uncultured Bacteroidetes bacterium	GQ870456
8 methanogenic endosymbionts	AJ132648-55
Methanoplanus endosymbiosus	AB370248, FR733674
Endosymbiont 'TS1' of *Trimyema compressum*	AB118591
Metopus palaeformis endosymbiont	M86386
Uncultured archeon clone BrCIArc	JF327423
41 Zoochlorellae	EF030554-62, 65-67, FN298917-25, EF044275, EU281549, EF589816, AB206546-50, AB506070-1, AB219527, AY876292, AB191205-7, AB162912-7
9 Cryptophytes	HQ226709, 13, 15, HQ226831, HQ226597, 99, AB4717788-9, DQ452092

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Chloroplast symbiosis in a marine ciliate: ecophysiology and the risks and rewards of hosting foreign organelles

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Simultaneous use of both heterotrophic and autotrophic metabolism ("mixotrophy") is common among protists. *Strombidium rassoulzadegani* is a planktonic mixotrophic marine ciliate that saves chloroplasts from its algal food and obtains a nutritional subsidy via photosynthesis. Cultures from the northeast, northwest, and southwest Atlantic Ocean show similar numerical response parameters (maximum growth rate, food concentration at which growth is half its maximum, and threshold food concentration for growth), and some isolates have been maintained *in vitro* for over 3 years. This ciliate grows equally well when fed on the green alga *Tetraselmis chui* (strain PLY429) or the cryptophyte *Rhodomonas lens* (strain RHODO). It appears to be an obligate mixotroph, requiring both food and light to achieve positive growth, when feeding on either of these algae. However, it has also been grown for several weeks (>10 generations) heterotrophically on the dinoflagellate *Prorocentrum minimum* (strain EXUV) during which it grows better in dark than in light. In this paper, we review the ecology of *S. rassoulzadegani*, discuss some aspects of its photo- and feeding physiology, and speculate on benefits and costs to the ciliate of chloroplast symbiosis.

Keywords: mixotrophy, *Strombidium*, oligotrich, chloroplast, photosynthesis, kleptochloroplast

INTRODUCTION

Oligotrich ciliates are among the most abundant small zooplankters in the sea. As grazers of primary production and food items for metazoan plankton they constitute an important link in the ocean's planktonic food web (Perez et al., 1997; Smetacek, 1981; Verity et al., 1999; Dolan et al., 2002; Olson and Strom, 2002; Ota and Taniguchi, 2003; Urrutxurtu et al., 2003). Early observers of oligotrichs sometimes reported colored bodies or other algal-like inclusions that were assumed to be the remains of ingested food (Calkins, 1901; Lohmann, 1908; Kahl, 1930). With the advent of fluorescence and electron microscopy, however, it was noted that some oligotrichs contain intact chloroplasts from ingested algae (Laval-Peuto and Febvre, 1986; Laval-Peuto et al., 1986; McManus and Fuhrman, 1986; Jonsson, 1987; Stoecker et al., 1987; Laval-Peuto and Rassoulzadegan, 1988) and subsequent work has shown that these chloroplasts remain functional within the cytoplasm for days to weeks, providing a photosynthetic subsidy to the ciliates' metabolism (Jonsson, 1987; Stoecker et al., 1988b; Putt, 1990). This mode of living has been referred to as kleptoplasty (stealing chloroplasts), or chloroplast "symbiosis," "sequestration," "retention," or "enslavement." It represents a kind of mixotrophy (having both auto- and heterotrophic nutritional modes) and appears to be common in ciliate assemblages in both oligotrophic and eutrophic environments, with mixotrophs comprising roughly 30% of all ciliates in the plankton (Stoecker et al., 1987; Perez et al., 1997; Dolan et al., 1999).

Concomitant with the discovery that many oligotrich ciliates harbor intact chloroplasts retained from ingested food was the recognition that ciliates and other microzooplankton are important trophic intermediaries (Gifford, 1988, 1991; Montagnes et al., 1988). It was thus quickly appreciated that mixotrophic ciliate photosynthesis could be an important carbon flow in planktonic food webs of marine waters, especially that in the microplankton size class (Stoecker et al., 1987).

In a recent review, Stoecker et al. (2009) categorized most oligotrich ciliate mixotrophy as a "persistent/obligate" arrangement, i.e., for a given mixotrophic species, retained chloroplasts are almost always present, and the ciliate cannot grow in the dark or on a diet of aplastidic cells, even if food is plentiful (Stoecker et al., 1988b). Because the oligotrichs are in general fastidious organisms that are difficult to maintain in culture for long periods of time (Gifford, 1985), experimental approaches to understanding the incorporation, maintenance, and management of retained chloroplasts have been limited to short durations. Comprehensive studies of food preferences and contributions of photosynthesis to the ciliate energy budget have been limited [cf. review in Johnson (2011)]. In a number of cases, it has been established that incorporation of inorganic carbon may account for enough energy to meet the maintenance respiratory requirements of the ciliate (Stoecker et al., 1988b; Putt, 1990) but there are currently insufficient data to support this as a generalization.

Persistent/obligate chloroplast retention has been viewed as a kind of symbiosis in other protists and in metazoa (Trench, 1969; Lopez, 1979). In the sense of "living together," the intact functional chloroplast and its ciliate host are indeed involved in a symbiotic relationship. However, since the chloroplast is from a genetically dead alga, its fitness when sequestered is zero and the relationship provides no apparent evolutionary benefit to the

alga. From the point of view of the ciliate, however, the symbiotic nature of the relationship is apparent in the contribution of the chloroplast's photosynthesis to its metabolism. What is not apparent is whether there are any costs to the ciliate of this arrangement that would make it a true evolutionary tradeoff in the same way that maintenance of zooxanthellae by corals or other forms of symbiosis are (Muller-Parker and D'Elia, 1997; Yakovleva et al., 2009).

The purpose of this paper is to review some features of chloroplast retention in the oligotrich *Strombidium rassoulzadegani*. Unlike many of its fellow mixotrophic oligotrichs, this species is quite amenable to cultivation and isolates have been maintained in the laboratory for several years. We will review the distribution of this species, discuss its cultivation both hetero- and mixotrophically, and report on some aspects of its growth and photosynthesis. In this context, we will also speculate on the costs of this form of mixotrophy.

MATERIALS AND METHODS

ISOLATION AND CULTIVATION

S. rassoulzadegani is reliably found in tide pools in temperate regions, being most abundant during summer. It has grass-green chloroplasts. Usually, a prominent red eyespot located in an apical bump at the anterior of the cell can be observed. For isolation, we either picked individuals with a drawn capillary pipette or enriched with the green microalga *Tetraselmis chui* (strain PLY429). This ciliate grows well on f/2 medium (Guillard and Ryther, 1962) in six-well plates (volume about 7 ml) at a salinity of 30, temperature of 19°C and a 12:12 light:dark cycle at 50–100 μmol $m^{-2}s^{-1}$. It appears to be euryhaline and eurythermal (data not shown). For larger volumes, we used flat tissue culture flasks and maintained a level of medium that resulted in the same surface area to depth ratio as in the well plates. Every time we have brought this species into culture, we verified its identity by comparing its internal transcribed spacer (ITS) sequence to that of the original isolate. It is curious that despite the observed ITS diversity of green tidepool ciliates *in situ* (Katz et al., 2005), *S. rassoulzadegani* is the only one that has been successfully cultivated for any length of time, to our knowledge.

Growth at different food concentrations was evaluated by placing 10–15 ciliates from well-fed cultures into wells at various food levels, in triplicate. Ciliates were first acclimated to food concentrations for 12–24 h, then transferred to new wells at each concentration. Growth was measured as change in ciliate abundance over 3 days, assuming exponential growth. Numerical response curves (growth rate, μ, versus food concentration) were fit to a Michaelis-Menten function with the addition of a threshold parameter (food concentration at which net growth is zero; Montagnes et al., 1996):

$$\mu = \mu_{max}[C - t]/(K + [C - t])$$

where μ is specific growth rate (d^{-1}), μ_{max} is the maximum growth rate, C is food concentration, K is the food concentration at which μ is half of μ_{max}, and t is the threshold food concentration. Curves were fit iteratively using SigmaPlot software.

To evaluate the effects of light level on growth, we exposed cultures of *S. rassoulzadegani* to various light levels using a fluorescent light table and neutral density film. Cultures grown at 100 μmol $m^{-2}s^{-1}$ were transferred to wells at saturating food concentrations (2×10^4 cells ml^{-1}) and continuous light levels from 0 to 200 μmol $m^{-2}s^{-1}$. Growth was measured over 3d and calculated assuming exponential growth. There was no initial acclimation period at the lower light levels because the resulting slower growth would have differentially aged the ciliates' chloroplasts and we wanted the chloroplast status to be uniform across all light levels at the start of the experiment.

AUTOTROPHY

To evaluate inorganic carbon uptake, we adapted the ^{14}C uptake method of Skovgaard et al. (2000). Ciliates that had been grown on the chlorophyte *Tetraselmis chui* (PLY429) were picked with a drawn pipette, rinsed in filtered seawater (FSW), and placed into six 20 ml scintillation vials containing 0.5 ml of FSW at a final count of 20 cells per vial. We also prepared six FSW controls and six vials of its food alga at 2×10^4 cells ml^{-1} so that we could compare algal and ciliate photosynthetic rates. We then spiked the algae, algae-free ciliates, and FSW controls with $NaH^{14}CO_3$ to a target final activity of 0.5 mCi ml^{-1}. To measure activity, 100 μl samples were taken from the FSW controls before and immediately after the $NaH^{14}CO_3$ addition, added to 200 μl of phenylethamine, an organic base, and measured with a liquid scintillation counter (LSC). Half of the experimental vials were incubated in the light (100 μmol photons $m^{-2}s^{-1}$) and half in the dark for 4 h. After incubation, vials were acidified and dried down to remove unincorporated inorganic carbon, leaving behind the carbon fixed by the ciliates. Samples were then re-suspended in 0.5 ml deionized water and activity was measured. The total inorganic carbon in the medium was determined using the method described in Parsons et al. (1984). Carbon incorporation rates (P) were calculated as

$$P = \frac{(DPM_L - DPM_D) \times W}{DPM_{t=0} \times t}$$

where P is in units of ngC ml^{-1} d^{-1}, DPM are disintegrations per minute in light (L), in dark (D), and at initial time ($t = 0$); W is the inorganic carbon concentration in the medium, and t is incubation time. Knowledge of cell concentrations and chlorophyll and carbon content were used to convert to rates per cell and turnover (d^{-1}).

PHOTOTAXIS

To evaluate phototaxis, we used a modified flat capillary assay (Levandowsky et al., 1984). Ciliates fed for several days on various algae were picked by drawn pipette in batches of 10 and placed in capillaries 50 mm long by 8 mm wide by 0.4 mm deep. The capillaries were shaded for one half their length with aluminum foil. Ciliates were initially placed in the dark half of the capillary and the capillary was placed on the stage of a stereomicroscope with the substage light turned on. After 2 min, the proportion of ciliates that had migrated to the light was measured. The capillary was then turned end-to-end and the foil cover moved to the other half, returning the ciliates from the lighted half back to darkness

for the next trial. For each food alga, we conducted five trials. Ciliates were also examined by compound light microscopy for maintenance of an eyespot on different foods.

SHORT-TERM LIGHT/DARK GROWTH AFTER ACCLIMATION

We performed several experiments to evaluate the degree to which mixotrophy is obligate in *S. rassoulzadegani* by comparing growth on several algal foods under light and dark conditions. In the first experiment, we compared growth on *Tetraselmis chui* (PLY429), *Rhodomonas lens* (RHODO), *Isochrysis galbana* (TISO), and *Prorocentrum minimum* (EXUV) in light and dark incubations. Ciliates were transferred from cultures grown on PLY429 into six-well plates containing each food at subsaturating levels (c. 100 cells/ml) and acclimated in the light at 19°C for 3 days. At the end of that time, the ciliates showed signs of color change (e.g., red for RHODO and brown for EXUV and TISO), but still contained some green chloroplasts. Ciliates were transferred to new wells containing the same algae they were acclimated to, at 10^4 cells ml^{-1}. For each food, there were two wells containing ciliates and one control well with algae only to verify that feeding took place. For each food treatment, we made two plates. One was incubated in the dark (wrapped in two layers of aluminum foil), and the other in the light (50 μmol $m^{-2}s^{-1}$). After 3 days, all wells were preserved with Lugol's iodine and ciliates and algae were counted.

LONG-TERM LIGHT/DARK GROWTH WITHOUT ACCLIMATION

A second experiment to evaluate heterotrophic growth on EXUV differed in that it did not include an initial acclimation period so that the transition from one food type to another could also be evaluated for light- and dark-incubated cells. This experiment also extended for a longer period, to evaluate sustained heterotrophic (dark) growth. Ciliates were transferred from PLY-grown cultures into triplicate wells containing EXUV at 10^3 cells ml^{-1} and incubated under light or dark conditions at 19°C. Food was added after 2 days to maintain saturating concentrations; after 5 days, five ciliates from each well were transferred to new wells at the same food and light conditions. The remaining ciliates were preserved and counted. This procedure was repeated at four successive intervals, whose lengths were 4, 4, 6, and 7 days, respectively. After 26 days, two of the dark wells suffered 100% mortality, so fresh triplicate light and dark wells were made from the survivors in the third dark well and followed for another 4 days.

FOOD SWITCHING EXPERIMENT

To evaluate survival and growth during the transition from light to dark, we performed a third experiment using the two algae that have consistently supported long-term culture of *S. rassoulzadegani*. Ciliates grown in the light for >3 weeks on RHODO or PLY429 were transferred to wells containing the other alga or an equal mixture of the two and incubated for 2 days under either light (50 μmol $m^{-2}s^{-1}$, 12:12) or dark conditions. Growth was measured by changes in abundance. Differences in responses to changes in food under light or dark conditions were evaluated by non-parametric Two-Way ANOVA performed on ranks (Sokal and Rohlf, 1995).

CARBON AND CHLOROPHYLL CONTENT

Cell volumes of ciliates and algae were calculated from measurements of linear dimensions of 10 cells each. Ciliate and algal carbon contents were estimated using volume to carbon relationships from Menden-Deuer and Lessard (2000). To measure chlorophyll content, *S. rassoulzadegani* cells were grown to high concentrations on a diet of PLY429 at concentrations that were saturating for growth (>10^4 cells ml^{-1}). We then separated the ciliates from their algal food using the ciliate's phototaxis. Ciliates were collected on glass fiber filters (Whatman GF/F) and extracted overnight in 90% acetone. Chlorophyll content was calculated from fluorescence.

RESULTS

ISOLATION AND CULTIVATION

To date, we have isolated *S. rassoulzadegani* from tidepools in Scotland, CT, USA, and ME, USA. We also isolated it from shallow water at a beach near Sao Sebastiao, Brazil. The initial isolate, from Connecticut, was grown on swarmer cells (zoospores and/or gametes) from the green macroalga *Ulva sp*., but we have subsequently cultivated it mostly on the small green alga *Tetraselmis chui* (strain PLY429). It also grows well on the cryptophyte *Rhodomonas lens* (strain RHODO). Identity of all isolates was verified by amplification and sequencing of a portion of the ITS region of the ribosomal gene (Katz et al., 2005; McManus et al., 2010).

We compared growth on different foods and among different isolates using numerical response data (intrinsic growth rate vs. food concentration) fit to a Michaelis–Menten function with an added parameter to account for threshold feeding at low concentrations. A summary of 16 experiments on five different isolates is given in **Table 1**. In all cases, the food was the same (PLY429), and experiments were conducted at 19°C and 100 μmol $m^{-2}s^{-1}$ light on a 12:12 light: dark cycle. Maximum growth rates averaged 1.32 ± 0.42 (sd) d^{-1}. The half-saturation (K) and threshold (t) parameters averaged 2300 and 440 cells ml^{-1}, respectively, but were more variable, with coefficients of variation >100%. This is likely due in part to the difficulty of estimating parameters where the curve is changing steeply (K) or where food concentrations are very low (t).

We have had some isolates in culture for >3 years, but eventually all of the cultures die out, probably due to accumulated genetic defects and genetic drift in small laboratory populations (Bell, 1988; Montagnes et al., 1996). However, we found

Table 1 | Means and standard deviations for three parameters of the modified Michaelis-Menten equation fit to the data on growth vs. food concentration.

Parameter	Units	Mean	SD	*n*
μmax	d^{-1}	1.32	0.42	16
K	cells ml^{-1}	2333	2747	16
t	cells ml^{-1}	434	633	16

The data are from 16 experiments conducted on four isolates, from New England USA (three isolates), Brazil, and Scotland. In all cases, the food organism was Tetraselmis chui (PLY429).

no clear trend of decline in μ_{max} with culture age. Even after 2 years in culture, our Maine (ME03) isolate grew at the same maximal rate as a freshly isolated one from Connecticut (CT05; **Figure 1**).

Strombidium rassoulzadegani grows very well on the green alga PLY429 and the cryptophyte RHODO (**Table 2**). It can be maintained for days to weeks on some other algae but we have never successfully cultured it for longer periods on them. When fed the dinoflagellate EXUV, or the prymnesiophyte TISO, the ciliate can be seen to ingest these cells and grow, gradually losing its green chloroplasts. After a few weeks at most, however, the cultures inevitably die out. A mixture of EXUV and TISO did not improve survival, and we never achieved growth on diatoms. Because the ciliates have been observed in field collections with green chloroplasts, we tried several other algae with green or yellow-green chloroplasts, including the tidepool-dwelling *Dunaliella tertiolecta* (DE) and the Eustigmatophyte *Nannochloropsis sp*. (UTEX2341), but could not achieve reliable growth (**Table 2**).

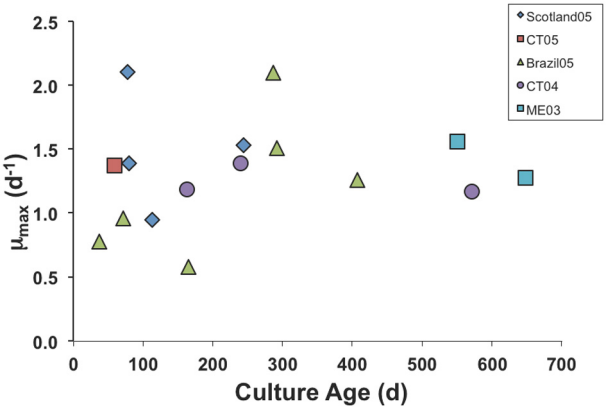

FIGURE 1 | Maximum growth rate parameter in numerical response curves for various isolates of *S. rassoulzaegani* fed *Tetraselmis chui* (PLY429), as a function of time since isolated. Strain designations indicate isolation locale (CT, Connecticut USA; ME, Maine USA) and year of isolation.

The numerical responses of ciliates grown on PLY, RHODO, or mixed RHODO/PLY were similar (**Figure 2**). In this experiment, values for the threshold parameter were not distinguishable from zero and K, the concentration where growth is half its maximum, ranged from 11 to 556 μgCl^{-1}. μmax was highest on the mixed food at 1.54 d^{-1}, but these parameters can be variable, and the difference was not statistically significant (**Table 1**). One feature of this experiment that is consistent in comparisons of these two foods is that the ciliate saturates more quickly on PLY429, suggesting that it only needs a small supply of fresh chloroplasts to maintain maximum growth rate on this food.

The numerical response of *S. rassoulzadegani* to variations in light intensity is shown in **Figure 3**. Because acclimation at low intensities would have resulted in differential aging of the chloroplasts (ciliates acclimated at subsaturating light would have contained older chloroplasts due to slower growth), we transferred the ciliates from exponentially-growing cultures at high food concentrations directly into the experimental wells. Thus, there was likely a carryover based on previous food conditions, and positive growth at zero light probably indicates this. The data show that food-replete ciliate growth becomes light saturated at approximately 50 $\mu mol\ m^{-2}s^{-1}$.

CARBON AND CHLOROPHYLL CONTENT

S. rassoulzadegani has about 60 times the carbon content of its algal food (PLY429), but more than 100 times the chlorophyll content (**Table 3**). When cells were picked individually, rinsed in FSW, and exposed to ^{14}C bicarbonate, they took up inorganic carbon in the light at rates per unit chlorophyll that were about half that of the algae. Because of the higher chlorophyll content, however, the turnover of C in ciliate and alga were about equal (0.13 and 0.14 d^{-1}, respectively; **Table 3**).

SHORT-TERM LIGHT/DARK GROWTH AFTER ACCLIMATION

In the experiment in which ciliates were acclimated in the light to TISO, EXUV, PLY429, or RHODO for 3 days, then transferred to

Table 2 | Ability of different algae to support growth in the oligotrich ciliate *Strombidium rassoulzadegani*.

Algal food tested	Strain	Class	Result
Tetraselmis chui	PLY429	Chlorodendrophyceae (Chlorophyta)	excellent growth; L >> D
Rhodomonas lens	RHODO	Cryptophyceae	excellent growth; L >> D
Prorocentrum minimum	EXUV	Dinophyceae	inconsistent growth, but L ≤ D
Isochrysis galbana	TISO	Prymnesiophyceae	inconsistent growth L, no growth D
Thalassiosira pseudonana	3H	Bacillariophyceae	no growth L or D
Thalassiosira weissflogii	Tweis	Bacillariophyceae	no growth L or D
*Nannochloropsis sp.**	UTEX2341	Eustigmatophyceae	no growth, only L tested
Stichococcus bacillaris	StichoGSB	Trebouxiophyceae (Chlorophyta)	no growth, only L tested
Chlorella autotrophica	580	Trebouxiophyceae (Chlorophyta)	no growth, only L tested
Dunaliella tertiolecta	DE	Chlorophyceae (Chlorophyta)	no growth, only L tested
mixed diet	TISO + EXUV	Dinophyceae + Prymnesiophyceae	no growth, survival c. 1 week, only L tested

*Class designations follow www.algaebase.org. Strain designations are from the Milford Microalgal Culture Collection. L, light (12:12 cycle, ~100 $\mu mol\ m^{-2}s^{-1}$); D, dark. *Also called Chlorella minutissima, but see (Gladu et al., 1995).*

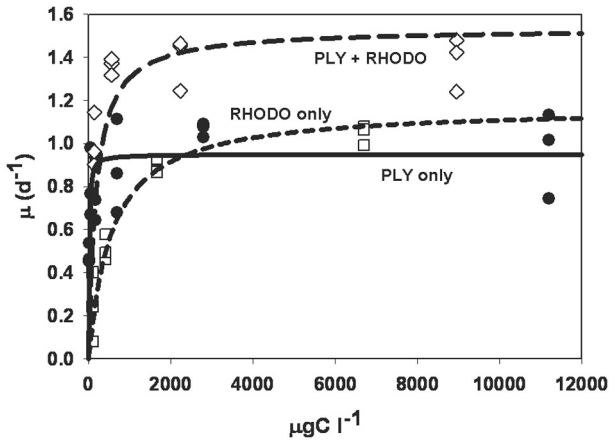

FIGURE 2 | Numerical response curves for *Strombidium rassoulzadegani* cultured on the chlorophyte PLY429 and transferred to a diet of PLY429, the cryptophyte *Rhodomonas lens* (RHODO) or a mixture of the two. Curves are fit to the Michaelis-Menten function $\mu = \mu_{max}$ [C−t]/(K + [C−t]), where μ is per capita growth rate, C is food concentration, K is the half-saturation constant, and t is a threshold below which growth is <0. For all three diets in this case, thresholds were not distinguishable from zero. The RHODO-only diet had a higher K than PLY-only or the mixed diet. In this case, the mixed diet appeared to give a higher maximum growth rate than either sole diet.

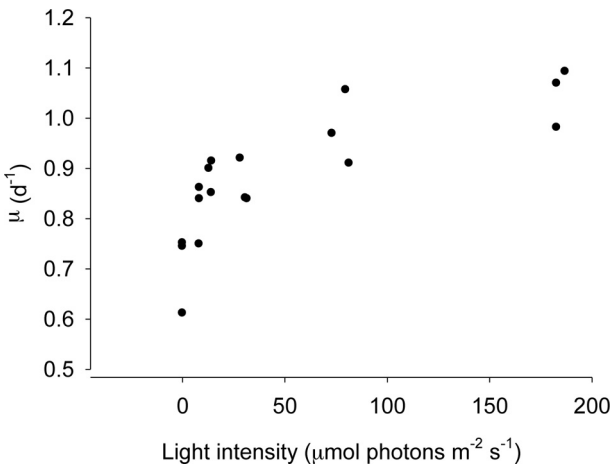

FIGURE 3 | Growth of *Strombidium rassoulzadegani* as a function of light intensity. Ciliates were grown at 100 μmol $m^{-2}s^{-1}$ and transferred to different light intensities without acclimation, ensuring that all chloroplasts were the same age, at a saturating food level of 2.5×10^4 PLY429/ml.

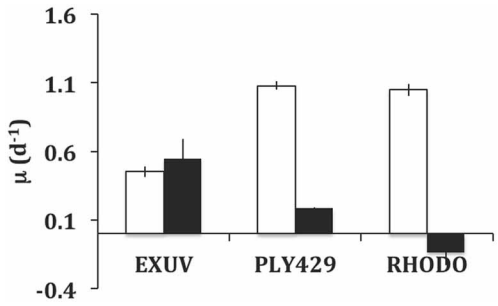

FIGURE 4 | Comparison of growth in light (12:12 cycle) vs. dark (continuous) for *Strombidium rassoulzadegani* fed one of three microalgae: *Prorocentrum minimum* (EXUV), *Tetraselmis chui* (PLY429), or *Rhodomonas lens* (RHODO). Error bars are ranges of duplicates.

light and dark treatments for 3 days of growth, only the cells on TISO showed 100% mortality. The other three algae supported growth (**Figure 4**) and there was net grazing of all algae (higher algal net growth in ciliate-free controls), including TISO, under both light and dark conditions. Ciliates on PLY429 showed much higher growth in light, compared to the dark treatment, and on RHODO showed net mortality in the dark, compared to the light, where growth was the same as on PLY429. The ciliate grew less well on the dinoflagellate EXUV than on the other two algae in the light, but better in the dark, with no difference between light and dark treatments.

LONG-TERM LIGHT/DARK GROWTH WITHOUT ACCLIMATION

In the longer term experiment with the dinoflagellate EXUV as food, we were able to grow *S. rassoulzadegani* for 30 days (**Figure 5**). During the initial transition from PLY-grown culture (5-days), the ciliate showed higher growth in light than in the dark, probably due to the fact that it was still using PLY429 chloroplasts. From day 5 to day 10, however, growth in the dark increased and growth in the light declined. Growth remained higher in the dark for the remainder of the experiment except that two of the three dark replicates suffered 100% mortality between days 19 and 26. After 26 days, the ciliates in the light treatment increased their growth rates, but growth was still slower than in the dark.

FOOD SWITCHING EXPERIMENT

We examined the growth responses of the ciliates to the transition between its two preferred foods, PLY429 and RHODO, and between light and dark conditions, during a 2 day experiment (**Figure 6**). Ciliates grown on RHODO grew better in light than

Table 3 | Volume, carbon and pigment contents, and photosynthetic incorporation of ^{14}C into the ciliate *Strombidium rassoulzadegani* and its food, *Tetraselmis chui* PLY429 (±standard deviation, where indicated).

	Volume μm^3 $cell^{-1}$	ngC $cell^{-1}$	pg chla $cell^{-1}$	pgC $cell^{-1}h^{-1}$	pgC pg $chl^{-1}h^{-1}$	d^{-1}
S. rassoulzadegani	33,510 ± 2800	6.50 ± 0.53	137 ± 19	35.9 ± 6.6	0.26 ± 0.05	0.13 ± 0.02
T. chui PLY429	781 ± 199	0.112 ± 0.03	1.33 ± 0.72	0.67 ± 0.17	0.50 ± 0.13	0.14 ± 0.04

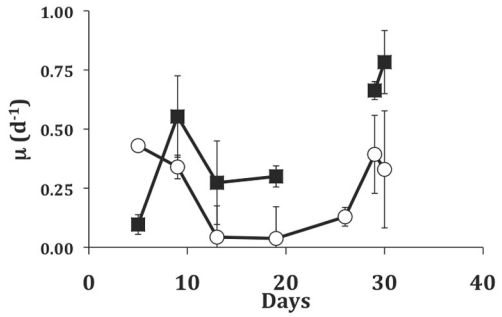

FIGURE 5 | Growth of *S. rassoulzadegani* on a diet of the dinoflagellate *Prorocentrum minimum* (EXUV) when incubated in dark (closed squares) or light (open circles). Symbols represent means of growth rate in the preceding interval, with standard deviations of triplicates. The gap in the dark treatment occurred when two of three replicates suffered 100% mortality, so the last two points in the dark treatment are ciliates transferred from the surviving well.

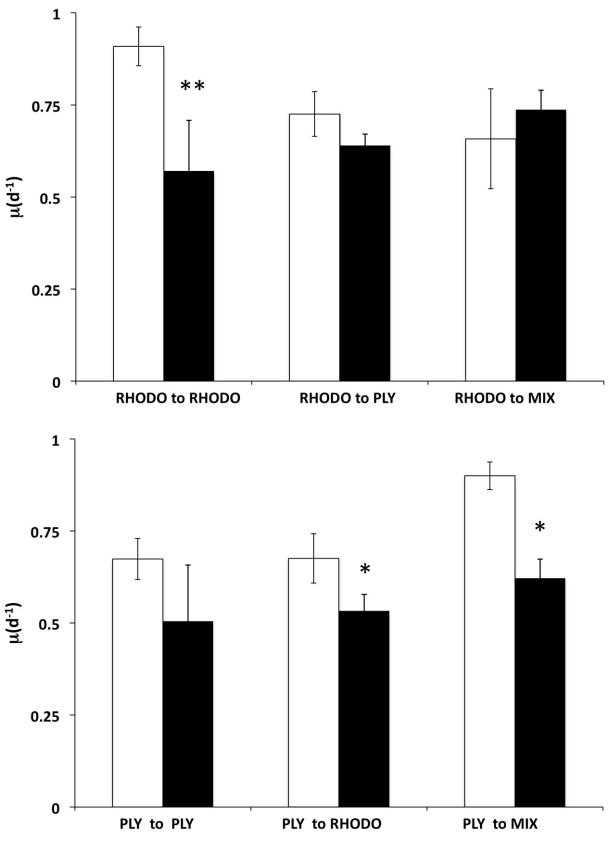

FIGURE 6 | Effect of switching food sources on light vs. dark growth. Ciliates cultured on *Rhodomonas lens* (RHODO, top panel) were transferred to RHODO, PLY, or an equal mixture of the two. Ciliates cultured on *Tetraselmis chui* (PLY, bottom panel) were likewise transferred to the PLY, RHODO, or a mixture. Means with standard deviations of triplicates. Significant differences between light and dark treatments indicated by * and ** for $P < 0.05$ and 0.01 (*t*-test), respectively.

in dark when kept on RHODO, although there was apparently a carryover effect that allowed about one division in the dark. This is supported by the fact that cells in the dark treatments were significantly smaller than those in the light (19% and 43% less cell volume in the PLY to PLY and RHODO to RHODO treatments, respectively), even though they had similar division rates. When switched from RHODO to PLY, there was no difference between light and dark treatments. Non-parametric Two-Way ANOVA indicated no significant added variance for the light or food treatments, but there was a significant interaction effect of light × food ($P < 0.05$). For the ciliates grown on PLY429, growth was higher in light than in dark for all three foods ($P < 0.01$ for the light effect), but there was no effect of food treatment on growth, and no significant interaction. As in the previous experiment, growth rate increased in the transition from PLY429 to the mixed diet. The RHODO-grown cells did not increase growth rate on the mixed diet (**Figure 6**).

PHOTOTAXIS

Ciliates that were cultured on PLY429 and transferred for 5d to RHODO, EXUV, or TISO all showed strong positive phototaxis. Regardless of food alga, all five replicates had at least 7 out of 10 ciliates migrate into the light within 2 min. Assuming that non-phototactic cells would be randomly distributed in the capillaries and hence equally likely to be in the dark or the light, the probability of this result occurring by chance is 2^{-5}, or 0.031. Even after several weeks of cultivation on RHODO, during which the ciliate lost its eyespot pigments, strong positive phototaxis was still retained (data not shown).

DISCUSSION

We originally discovered *S. rassoulzadegani* as a sequence of the ITS region of the ribosomal gene (ITS1-5.8S-ITS2) from a clone library of samples collected in tide pools on the Irish Sea and Galway Bay, Ireland (clade vii in Katz et al., 2005). In these field-collected samples of green tidepool ciliates, we found surprising hidden diversity, with sequences differing by up to 16% at this locus (Katz et al., 2005). Subsequently, we isolated and cultivated clade vii from samples collected in Scotland, Brazil, and several sites in New England, USA. After cytological staining and comparision with previously-reported ribosomal gene sequences in GenBank, we named clade vii as the new species *S. rassoulzadegani* (McManus et al., 2010).

S. rassoulzadegani is typical of mixotrophic oligotrichs in requiring a constant supply of fresh chloroplasts from ingested food in order to maintain photosynthetic capability. Complete turnover of chloroplasts takes about 48 h (Schoener and McManus, in review). *In situ*, it has green chloroplasts, like its congener *S. oculatum* and several other estuarine strombidiids (Jonsson, 1987; Stoecker et al., 1988a), and it likely feeds on the gametes or zoospores of green macroalgae (McManus et al., 2004).

In common with several other "green" strombidiids, *S. rassoulzadegani* has an apical eyespot that is likely used in phototaxis. Because both the putative natural prey (swarmer cells of green macroalgae) and green microalga on which we cultivated it contain eyespot pigments and a photosensing system, we initially

speculated that the source of the ciliate eyespot was also its food. While it appears that ciliates cultivated on the cryptophyte RHODO do lose their eyespots, their ability to orient toward light in a positive phototaxis was not diminished in our observations. There may be some shading of the ciliate photoreceptor (unknown at present) by the sequestered chloroplasts to compensate for eyespot loss, but this is speculative. In the context of mixotrophy's costs and benefits, the potential role of algal eyespot pigments in the ciliate's phototaxis deserves further study.

This ciliate grows equally well on the green PLY429 or the red RHODO (**Figure 2**), retaining both kinds of chloroplasts. It has been maintained for >3 years in the lab on PLY429. We did not observe any decline in physiology (as indicated by maximum growth rate) in long term isolates, though most of them die out within 2 years for reasons that are not known. Although use of cryptophyte chloroplasts has been shown in several other mixotrophic oligotrichs, it seems unusual that they would be interchangeable with those of green algae insofar as ciliate growth rate is concerned. The transition from red to green chloroplasts over 2d was nearly indistinguishable from that of green to red (**Figure 6**), except that RHODO-grown cells showed a significant interaction between food and light treatments when exposed to a new food (ANOVA results; **Figure 6**), whereas PLY-grown cells maintained about the same growth advantage in light vs. dark regardless of food (**Figure 6**). Given the very strong differences between light and dark growth on RHODO (**Figures 4**, **6**), we interpret this to indicate that ciliates switching from RHODO to another kind of chloroplast, in this case PLY429, suffer some disadvantage in comparison to PLY-grown cells, which retain their PLY429 chloroplasts longer when transferred to RHODO (Schoener and McManus, in review). This suggests a stronger integration of green chloroplasts when the ciliates are feeding on green algae. More observations of growth rate and growth efficiency in light and dark at low food concentrations may help to resolve this issue.

On either of these food organisms, *S. rassoulzadegani* appears to be a "persistent/obligate" mixotroph, *sensu* Stoecker et al. (2009), requiring both food and light for growth (**Figure 4**). It can, however, grow for days or weeks on some other algae, including the dinoflagellate EXUV (**Figures 4**, **5**). This is much longer than the period in which it can retain its green chloroplasts. Furthermore, it can grow as well or better in the dark compared to in the light on EXUV, suggesting that growth is fully heterotrophic. The transition from mixo- to heterotrophic growth sometimes fails, however, and there may be some conditions of food or ciliate growth cycle that prevent it. After several weeks of feeding on EXUV in the dark, growth rates were about 0.75 d^{-1}, similar to those of non-mixotrophic oligotrichs (0.5–1.4 d^{-1}; Gismervik, 2005). These results thus suggest caution in assigning "obligate" status to this kind of symbiotic arrangement unless exhaustive trials on other foods have been performed.

One interesting observation from the experiment in which *S. rassoulzadegani* was maintained for 30d on EXUV was that ciliates initially grew better in the light, undoubtedly due to the leftover chloroplasts from PLY429, but then grew much worse than the dark treatments, just surviving in the light (**Figure 5**). After about 2 weeks, growth rates in the light increased, but remained lower than those in the dark despite the fact that in the light treatment food algae were more abundant than in the dark treatments due to increased algal growth in the light. It may be that exposure to light suppresses digestion of the chloroplasts. Laval-Peuto and Febvre (1986) and Stoecker and Silver (1990) observed vacuoles containing deteriorating chloroplasts. These appeared to be autophagous and distinct from food vacuoles. They also were rare, suggesting that digestion of chloroplasts usually does not occur and old, non-functional chloroplasts may be egested rather than digested. We have not observed this directly, and starved ciliates appear to retain chloroplasts right up until death (Schoener and McManus in review). If the EXUV chloroplasts in this case are unsuitable for phototrophic use by the ciliate and somehow marked as immune from digestion in the light, they would become a liability and slow ciliate growth relative to that in the dark until they could be egested. We have not attempted to measure ^{14}C incorporation by ciliates grown on EXUV, but we speculate that those chloroplasts are not usable by the ciliate and hence slow its growth in the light. Much more work remains to be done on the intracellular signals that prevent digestion of chloroplasts in mixotrophic ciliates before this issue can be resolved.

The observation of slower growth in the light on EXUV raises the question of the benefits and potential costs of mixotrophy. It seems obvious that the ability to retain and use ingested chloroplasts is a strong benefit to the ciliate because photosynthetically-incorporated carbon would be an autotrophic subsidy for growth. In culture, mixotrophs like *S. rassoulzadegani* need relatively little food to achieve maximum growth (**Figure 2**) and have high growth efficiencies (Johnson and Stoecker, 2005; Schoener and McManus, in review). Dolan and Perez (2000) found evidence of reduced predation on mixotrophs, compared to heterotrophic ciliates, suggesting a second benefit of mixotrophy. Berger (1980) observed a similar phenomenon in *Didinium/Paramecium* predation, with algal symbiont-bearing *Paramecium* being half as susceptible to predation as those without symbionts. Other potential benefits, including removal of nitrogenous wastes or provision of oxygen, have been demonstrated in other symbioses (e.g., Finlay et al., 1996; Davy et al., 2002), but have not been documented yet in plastid-sequestering ciliate mixotrophs, to our knowledge.

The potential costs of mixotrophy are not well-known. In ciliates where mixotrophy is obligate, the light requirement obviously limits them to the photic zone. However, the observation of "obligate" mixotrophy in chloroplast-retaining ciliates has been based mostly on laboratory cultures, where diversity of food is limited. Studies on field-caught mixotrophs have revealed multiple chloroplast types in some species (Laval-Peuto and Febvre, 1986; Stoecker and Silver, 1987), and it seems likely that the diets of mixotrophs in nature are varied. Our finding that a ciliate that is apparently obligate when fed one alga could be maintained in the dark on another suggests that some switching between mixotrophy and heterotrophy may be possible, at least in this species and under some circumstances. The fact that heterotrophic growth was worse in the light suggests that ciliates with the capability for chloroplast sequestration may pay a price in growth when suitable chloroplasts are not available in the diet. Other possible costs of retaining chloroplasts, such as the increased

oxidative stress due to symbionts that has been observed in zooxanthellae-bearing coral larvae (Yakovleva et al., 2009), have not been studied in mixotrophic ciliates and deserve investigation.

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Endophytic bacterial community of a Mediterranean marine angiosperm (*Posidonia oceanica*)

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Bacterial endophytes are crucial for the survival of many terrestrial plants, but little is known about the presence and importance of bacterial endophytes of marine plants. We conducted a survey of the endophytic bacterial community of the long-living Mediterranean marine angiosperm *Posidonia oceanica* in surface-sterilized tissues (roots, rhizomes, and leaves) by Denaturing Gradient Gel Electrophoresis (DGGE). A total of 26 *Posidonia oceanica* meadows around the Balearic Islands were sampled, and the band patterns obtained for each meadow were compared for the three sampled tissues. Endophytic bacterial sequences were detected in most of the samples analyzed. A total of 34 OTUs (Operational Taxonomic Units) were detected. The main OTUs of endophytic bacteria present in *P. oceanica* tissues belonged primarily to Proteobacteria (α, γ, and δ subclasses) and Bacteroidetes. The OTUs found in roots significantly differed from those of rhizomes and leaves. Moreover, some OTUs were found to be associated to each type of tissue. Bipartite network analysis revealed differences in the bacterial endophyte communities present on different islands. The results of this study provide a pioneering step toward the characterization of the endophytic bacterial community associated with tissues of a marine angiosperm and reveal the presence of bacterial endophytes that differed among locations and tissue types.

Keywords: ***Posidonia oceanica*, seagrass-bacteria interaction, DGGE, endophytes**

INTRODUCTION

Bacteria are commonly found living endophytically within plant tissues (e.g., Hallmann and Berg, 2006). Endophytic bacteria, typically defined as those living inside plant tissues not harming the host plant (Schulz and Boyle, 2006), often promote plant growth by, for instance, providing nutrients or controlling plant pathogens through mutualistic bacteria–plant interactions (e.g., Hallmann and Berg, 2006; Ikeda et al., 2010; Li et al., 2011). Moreover, symbionts can also be pathogenic bacteria that, when present at high abundances, cause plant disease outbreaks. Information on composition and ecological roles of symbiotic bacterial communities abounds for terrestrial and freshwater plants, particularly for crop species (e.g., Ueda et al., 1995); however, the presence and relevance of symbiotic bacterial communities in marine plants remain unexplored.

Seagrasses are marine clonal angiosperms that evolved from freshwater angiosperm ancestors that colonized the marine environment in the Cretaceous (den Hartog, 1970). Despite the fact that seagrass flora is restricted to approximately 50–60 species, they develop lush and highly productive meadows, particularly in oligotrophic waters, along the coasts of all continents except Antarctica (Hemminga and Duarte, 2000; Short et al., 2007). Seagrass meadows are important global carbon sinks, enhance coastal biodiversity and prevent coastal erosion (Hemminga and Duarte, 2000; Orth et al., 2006). Bacterial communities play important roles in seagrass meadows, particularly in the recycling of materials (Hemminga and Duarte, 2000). However, information about bacterial communities associated with seagrasses is scant, with most studies focusing on bacterial communities in seagrass sediments (Cifuentes et al., 2000; Bagwell et al., 2002; Garcia-Martinez et al., 2009) or associated with plant surfaces (i.e., epiphytic bacterial community) above (Weidner et al., 2000; Jensen et al., 2007; Uku et al., 2007; Crump and Koch, 2008) or belowground (Garcia-Martinez et al., 2005). However, endophytic bacteria in seagrass (*Thalassia hemprichii, Cymodocea serrulata, Halodule uninervis, Syringodium isoetofolium*) tissues have been reported using optical microscopy (Kuo, 1993). *Clostridium glycolicum* has been isolated from the rhizoplane and deep cortex cells of *Halodule wrightii* (Küsel et al., 1999), a new species of the genus *Sulfitobacter* has been isolated from a homogenate of *Zostera marina* (Ivanova et al., 2004), and *Desulfovibrio zosterae* has been isolated from the surface-sterilized roots of *Z. marina* (Nielsen et al., 1999), indicating that endophytic bacteria occur in seagrass tissues.

Posidonia oceanica is the dominant seagrass species in the Mediterranean Sea. Although *P. oceanica* ranks among the slowest growing seagrasses (rhizome extension rates ranging from 1 to 6 cm yr^{-1} $apex^{-1}$, Marbà and Duarte, 1998), it develops meadows living for millennia (Mateo et al., 1997; Arnaud-Haond et al., 2012) and occupies an estimated 50,000 km^2 in the Mediterranean Sea. The unique environments found in and around *P. oceanica* tissues constitute niches well differentiated

from those in surrounding waters and sediments. Moreover, the millenary life span of *P. oceanica* clones suggest that endophytic bacteria can remain isolated within the *P. oceanica* tissues over extended periods of time, relevant for microbial evolutionary processes. Thus, it is likely that *P. oceanica* meadows harbor a distinct microbial community including previously undescribed species. Indeed, recent studies using culturing methods have described seven new bacterial species belonging to the genus *Marinomonas* isolated from *P. oceanica* (Espinosa et al., 2010; Lucas-Elío et al., 2011), supporting the idea of the existence of a distinct bacterial community associated with *P. oceanica*.

The interest in exploring the endophytic bacterial community of *P. oceanica* extends beyond that of exploring a potential biodiversity niche. The characterization of the microbes found inside the tissues of *P. oceanica* can offer significant clues about the health and ecology of *P. oceanica* meadows. Moreover, the number of disease outbreaks in the marine environment appears to be rising (Harvell et al., 1999). This trend is possibly facilitated by anthropogenic pressures (e.g., global movement of ballast waters by ships, Ruiz et al., 2000) and global warming (Harvell et al., 1999, 2002) as they may facilitate the occurrence of pathogens in areas with previously unexposed host populations. Symbiotic microorganisms can also play a key role in determining seagrass population dynamics as they can facilitate the uptake of elements like nitrogen, which can be limited in marine environments. The role of bacteria in sulfur cycling can also determine the health, and therefore the growth rates of marine angiosperms in marine sediments receiving high organic matter inputs. H_2S produced from decomposition of organic matter under anoxic conditions can intrude into seagrass tissues (Pedersen et al., 2004), with negative consequences for seagrass meristematic activity (Garcias-Bonet et al., 2008). Bacteria can, therefore, play a major role in the survival and growth of seagrass meadows. The characterization of the microbiota closely associated with *Posidonia oceanica*, such as endophytic bacteria, is a first step that may provide further insights into the complex interactions between bacteria and seagrass.

Here we describe the bacterial communities associated with surface-sterilized tissues (roots, rhizomes, leaves) collected in summer in 26 meadows of *Posidonia oceanica* around the 950 km of coast of the Balearic Islands (Western Mediterranean). We used DGGE (Denaturing Gradient Gel Electrophoresis) to analyze the community structure of endophytic bacteria in the plant tissues. The banding profiles derived were compared across locations, and dominant bands were sequenced to provide a first identification of bacterial endophytes of Balearic *P. oceanica*.

MATERIALS AND METHODS

SAMPLING STRATEGIES

Posidonia oceanica shoots were collected at 26 locations across the Balearic Islands (**Figure 1**) by SCUBA diving during the summers of 2005 and 2006. The plants were transported to the laboratory in

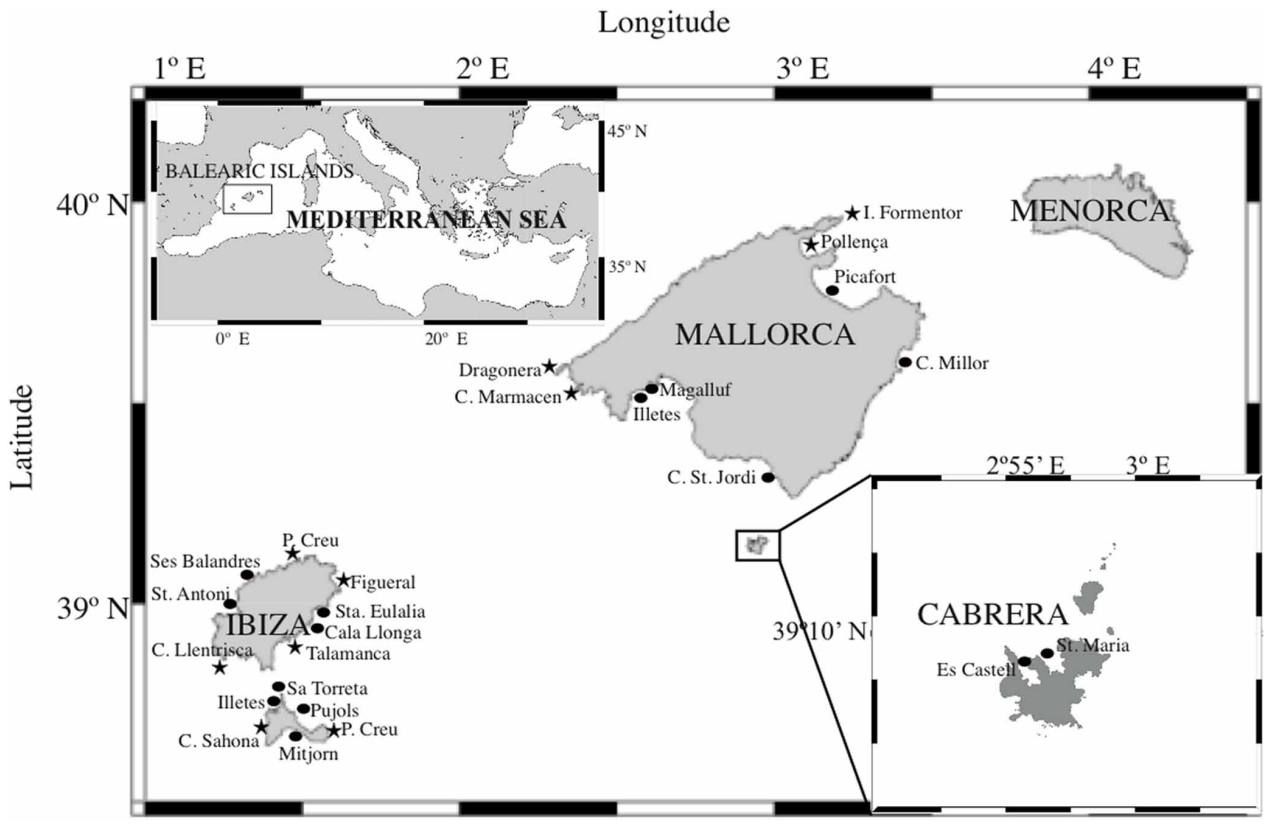

FIGURE 1 | Location of *P. oceanica* meadows sampled in summer 2005 (circles) and summer 2006 (stars).

seawater from the same location and processed immediately. The leaves, rhizomes, and roots from three shoots per meadow were separated and subsequently subjected to a surface-sterilization protocol adapted from Coombs and Franco (2003). Briefly, the protocol consisted of immersing each sample in 99% ethanol for 1 min, then in 3.125% NaOCl for 6 min, then in 99% ethanol for 30 s and finally washing gently with autoclaved seawater. These surface-sterilized samples were frozen in liquid nitrogen until nucleic acid extraction was performed.

NUCLEIC ACID EXTRACTION AND AMPLIFICATION

Surface-sterilized plant material (100 mg of fresh tissue) was ground with the help of a sterilized pestle. The total nucleic acid extraction was performed using a commercial kit specific for plant tissues (Partec®). Nucleic acid extracts were stored at -20°C until amplification. The DNA extract, containing plant and endophyte DNA when present, was amplified by standard PCR with primers 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) and 341F-GC containing a 40 bp GC clamp at the 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C/CC TAC GGG AGG GAG CAG-3′) specific for the bacteria domain (Muyzer and Smalla, 1998). Additional negative (no DNA) and positive (*E. coli* DNA) control reactions were run with each batch of PCR reactions. The PCR products were checked by electrophoresis on 1% agarose gels. For each sample, the products of several replicate reactions (minimum of 2) were pooled prior to DGGE.

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

The amplification products of the fragment of the 16S ribosomal RNA gene (1 μg of PCR product) were separated by DGGE in a 6% polyacrylamide gel containing a gradient of denaturants ranging from 40 to 70% (where 100% is 7 M urea and 40% formamide). Gels were run for 18 h at 150 V in 1X TAE (Tris-Acetate-EDTA) buffer at 60°C in a CBS Scientific Co., DGGE system. Following electrophoresis, the gels were stained with SybrGold for 30 min in the dark and photographed using a G:BOX imaging system (Syngene). All the detectable bands were excised and stored frozen in autoclaved MiliQ water at −20°C for further processing.

ANALYSIS OF DGGE PROFILES

The digital images of DGGE gels were analyzed by measuring the relative migration of each band, normalized to the migration of the 16S rDNA band corresponding to *Posidonia oceanica* chloroplasts, which were detectable on every sample. Additional DGGE gels containing replicates of PCR products already analyzed in different gels were run in order to facilitate comparisons across different DGGE gels. The bands with the same normalized migration distance were identified as the same Operational Taxonomic Unit (OTU), confirmed by sequencing of some bands.

Species accumulation curves (i.e., accumulated increase of the number of detected OTUs vs. number of samples) were constructed in R (R Development Core Team, 2011; http://www.R-project.org/) using package vegan (Oksanen et al., 2011) in order to check accuracy and representativeness of the sampling strategy and, therefore, of our results. Estimates of species richness (Chao, Jackknife, and Bootstrap) were obtained from accumulation curves using the function *specaccum* in package vegan.

A binary matrix (presence/absence) was constructed for all of the identified OTUs in order to determine the similarity among samples and locations. Using the information about presence/absence of each OTU in different tissues of *P. oceanica*, we have constructed weighted bipartite networks for each location studied to represent the endophyte–plant network of bacterial endophytes and *P. oceanica* tissues. In a bipartite network, there are nodes of two distinct types, and the edges connect only nodes of different kinds (Albert and Barabási, 2002; Newman, 2003). For the networks used in this study, one set of nodes was composed of the 34 detected OTUs, and the other set was composed of the tissue groups (roots, rhizomes, and leaves), totaling 37 nodes. Links between the two sets of nodes were drawn if an OTU was observed in a tissue, and the weights of these links were represented by the sum of the relative observations of each OTU. We call "relative observation of an OTU" the ratio between the number of observations of the OTU in the tissue and the number of replicates in each location studied.

Once the weighted bipartite networks for each location were constructed, we collapsed the networks of locations at the same island (Cabrera, Formentera, Ibiza, and Mallorca) in order to obtain the weighted bipartite network for each island. By collapsing the networks of all locations, we obtained the weighted bipartite network for the Balearic Archipelago.

We compared the bipartite networks of each island using the concept of the distance between networks with the same number of nodes, as described by Andrade et al. (2008). They used the shortest paths and the diameter of a pair of networks to give a quantitative and normalized value to represent the similarity between these networks. We adapted their method by using the weighted shortest path and weighted diameter in order to compare the similarity in the weighted bipartite network among islands.

We used a bootstrap strategy to examine the robustness of the network analysis. We randomly removed one node of the networks to be compared and computed the distance between them. After repeating this procedure for each node, we calculated the average of the distances computed for each pair of networks. This average was considered as the best estimate of the average distance between any pair of networks, and the procedure was repeated for each pair of networks (each pair of locations).

We used the Girvan–Newman algorithm (Girvan and Newman, 2002) to identify which nodes of the Balearic Island bipartite network were more densely grouped representing communities of endophytic microorganisms that tended to co-occur. Although the original Girvan–Newman algorithm was developed for unweighted, undirected networks, here we have adapted this algorithm to enable the community analysis of weighted networks, as suggested by Yoon et al. (2006).

The binary matrix was also used to generate a distance matrix based on Jaccard's coefficient as the basis for a non-metric multidimensional scaling (NMDS) diagram using package vegan in R. We performed an Analysis of Similarity (ANOSIM) using the vegan package (10,000 permutations), to test for the existence of differences in band patterns among tissue groups defined as

roots, rhizomes, and leaves. The R value generated by ANOSIM test indicates the magnitude of difference among groups, where an $R > 0$ indicates differences between groups and $R < 0$ indicates no difference between groups, because differences between groups are lower than differences within a group. The significance of ANOSIM results was tested using the Bonferroni correction as *post-hoc* test.

Finally, we performed an indicator species test (Dufrene and Legendre, 1997) using package labdsv (Roberts, 2010) in R software to identify those OTUs that are characteristics of each tissue and island. The indicator species are defined as the most characteristic species of each group, found mostly in a single group and present in the majority of the sites or samples belonging to that group.

SEQUENCING OF THE OTUs DETECTED IN DGGE

The detected and excised bands (OTUs) from the DGGEs were reamplified using the same pair of primers (907R and 341F-GC). The amplification products were cleaned and purified from primers and dNTPs by an enzymatic reaction with a mixture of Exonuclease I (1 U/reaction) and Alkaline Phosphatase (1 U/reaction) at 37°C during 60 min, followed by an enzyme denaturing step at 72°C for 15 min. The DNA was precipitated using isopropanol (66% final concentration), centrifuged (10,000× g, 15 min), washed with 66% isopropanol and resuspended in sterile water. The resulting DNA concentration was measured fluorometrically (Qubit®, Invitrogen) and 150 ng of the amplified product was used for the sequencing reaction using the reverse primer 907R. The sequencing was performed by Secugen, using the chemistry BigDye® Terminator v3.1. The sequences of about 500 bp were checked for existence of chimeras using the Bellerophon tool available at http://greengenes.lbl.gov and compared to the public DNA database of NCBI by using BLAST (Basic Local Alignment Search Tool) service at the National Center of Biotechnology Information (NCBI) web page (www.ncbi.nlm.nih.gov). Further validation of the phylogenic identity of the sequences was performed by aligning the sequences to those in the greengenes database (http://greengenes.lbl.gov) using ARB (Ludwig et al., 2004). A Neighbor Joining tree of full sequences of the closest relatives was constructed in ARB and the shorter DGGE sequences were added to that tree using the ARB parsimony interactive tool. Bootstrap values were also generated using the ARB interactive parsimony tool.

The sequences obtained in this study have been deposited in Genbank under the accession numbers JF292432 to JF292446.

RESULTS

A total of 34 different OTUs were identified in DGGE profiles from all plant tissue samples ($n = 186$). Rhizome samples ($n = 57$) and root samples ($n = 67$) hosted 28 different OTUs while leaf samples ($n = 62$) showed 24 different OTUs detectable by DGGE analysis. Thirteen rhizome samples (18.6%), five root samples (6.9%), and eight leaf samples (11.4%) did not show any band, except the band corresponding to the 16S rDNA of the chloroplast.

The species accumulation curves (**Figure 2**) confirmed that the sampling effort was adequate to characterize the bacterial community richness associated with *P. oceanica* tissues, as curves showed saturation (i.e., approached a plateau), suggesting that more intensive sampling was likely to yield only minor improvements in coverage. The Chao, Jackknife, and Bootstrapping estimates of species richness (**Table 1**) indicated that the percentages of OTUs detected in our DGGE gels accounted for 97–99.6% of the total community richness for all tissues sampled. Large coverage was estimated for all tissue classes, the percentages of detected richness varied between 95.6–99.6% (leaves), 93.4%–98.9% (rhizomes) and from 69.1 to 93.1% (roots) depending on the particular estimate used. Despite the relatively high numbers of OTUs found overall, individual samples of different tissues contained relatively low numbers of OTUs. Roots presented 3.56 ± 2.5 different OTUs (average $\pm$ SD) per sample, while only 2.46 ± 2.4 and 2.7 ± 2.5 OTUs were found in rhizomes and leaves, respectively.

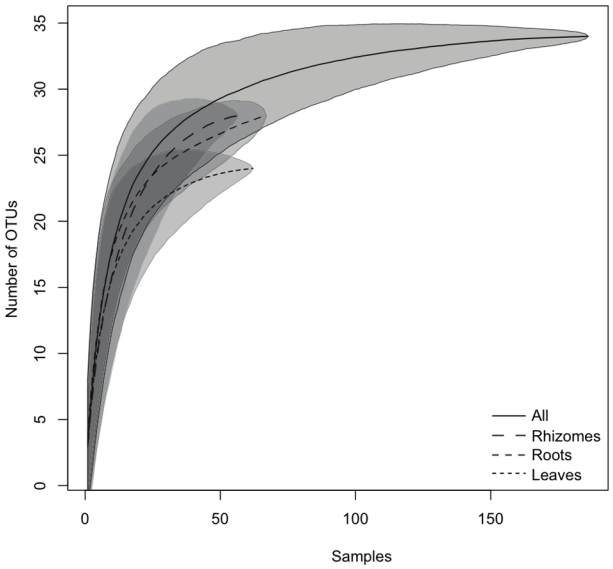

FIGURE 2 | Species accumulation curves of the endophytic bacterial community found in *P. oceanica* tissues. The lines indicate the averaged accumulated increase of detected OTUs vs. number of samples (10,000 bootstrap sampling replicates). The shadowed area indicates the standard deviation. The continuous line represents all the samples pooled together, and the dashed lines provide the values for the different tissues.

The bipartite network analysis showed differences in the band patterns among islands (**Figure 3**), where meadows located in Ibiza and Mallorca islands seemed to be the most similar among them (more than 97% of similarity). Conversely, the networks of Cabrera and Formentera islands were the most different (93–95% of similarity). The other pairs of islands (Ibiza–Formentera; Ibiza–Cabrera; Mallorca–Cabrera; Mallorca–Formentera) have endophyte–plant networks with 95–97% similarity among them.

The community analysis of the bipartite network of all the Balearic Islands studied, obtained by running the Girvan–Newman algorithm, and identified three different communities for each tissue type (**Figure 4**). The algorithm did not associate any community with the OTU 1.

Table 1 | Number of total OTUs detected in each tissue for all the samples tested; total number of OTUs estimated by different approaches: Chao, Jackknife and Bootstrapping; and percentage of the total estimated OTUs detected in the samples.

			Number of OTUs expected by			Percentage of sampled OTUs from the expected number by		
Groups	**N**	**Number of OTUs detected**	**Chao approach**	**Jackknife approach**	**Bootstrapping approach**	**Chao approach**	**Jackknife approach**	**Bootstrapping approach**
All	186	34	34.12 ± 0.44	34.99 ± 0.99	35.04 ± 0.98	99.65	97.16	97.03
Leaves	62	24	24.1 ± 0.38	24.98 ± 0.98	25.09 ± 1.28	99.59	96.06	95.64
Rhizomes	57	28	28.29 ± 0.68	29.96 ± 1.38	29.89 ± 1.67	98.97	93.44	93.67
Roots	67	28	40.5 ± 17.14	32.93 ± 2.61	30.08 ± 1.34	69.14	85.04	93.08

N = number of samples.

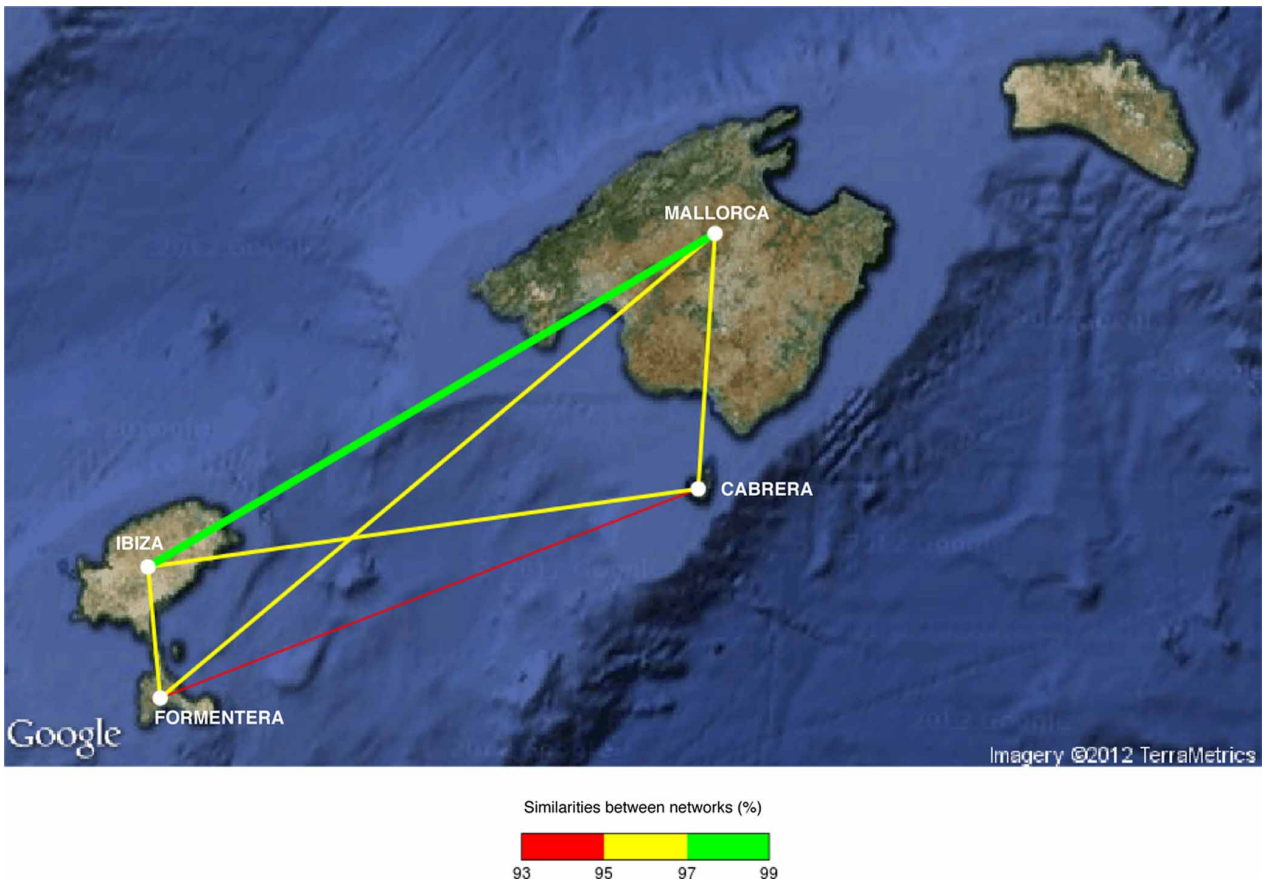

FIGURE 3 | Bipartite network analysis of endophytic bacterial community of *P. oceanica* tissues among islands. The nodes represent the islands. Thicker edges between two islands indicate larger similarity of the bipartite networks. Edges are also color-coded to indicate the percentage of similarity between two islands.

Although NMDS did not show clear differences among tissues (data not shown), ANOSIM test confirmed that band patterns among tissues were different with statistically significance, although these differences were small, suggesting other variables play a role in the endophytic bacterial composition of *P. oceanica* tissues. The band patterns obtained in DGGE analysis for root tissues were different from those obtained for rhizome and leaf tissues ($R = 0.201$, $P < 0.005$ and $R = 0.126$, $P < 0.005$, respectively) and greater than the differences obtained between band patterns in leaf and rhizome tissues ($R = 0.046$, $P < 0.05$).

Moreover, the indicator species analysis identified some OTUs characteristic of each tissue (**Table 2**), although the indicator values were low. According to indicator species analysis, two OTUs were associated with leaves, five OTUs were associated with rhizomes, and six OTUs were associated with roots.

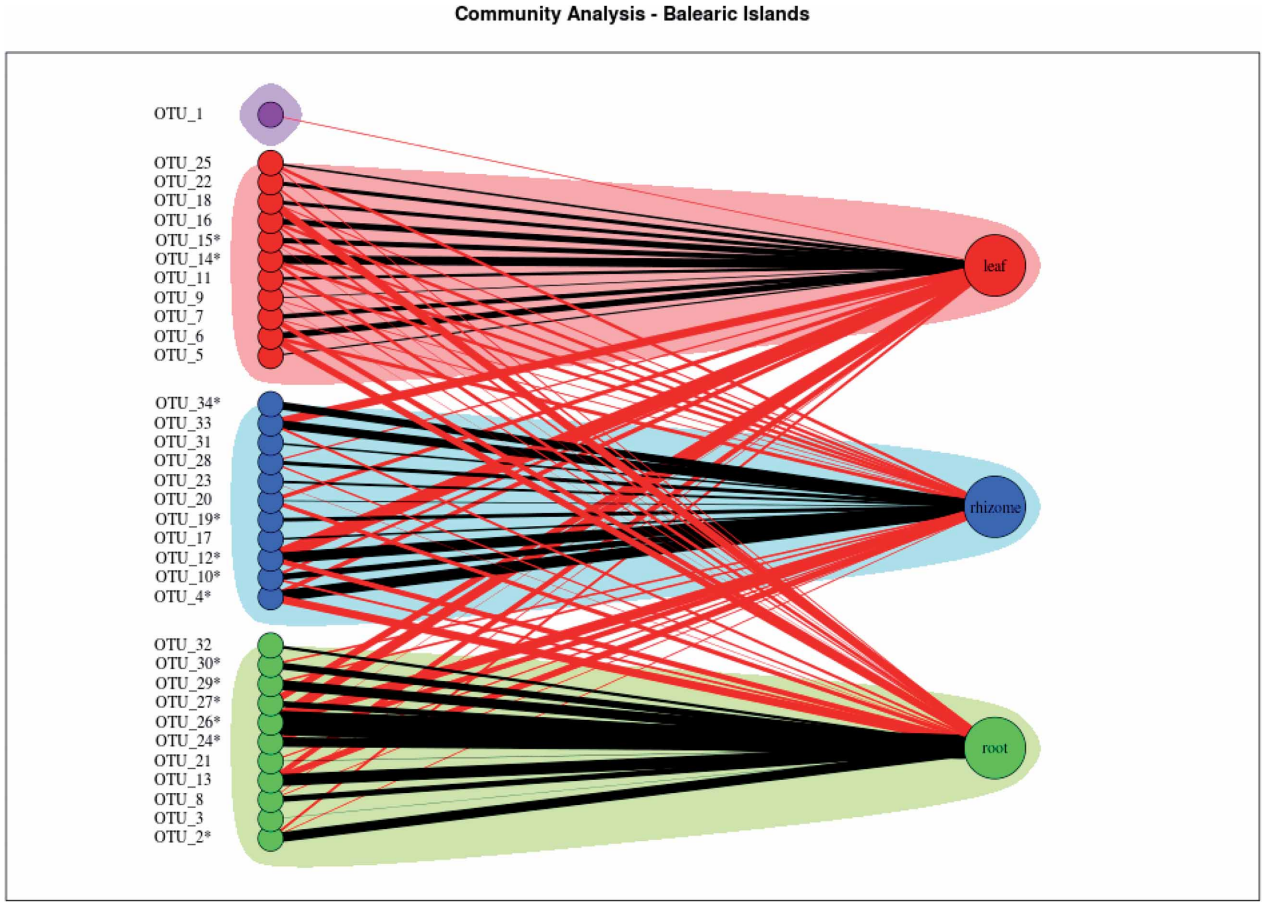

FIGURE 4 | Community analysis of the bipartite network of all the Balearic Islands studied. Shadowed areas indicate communities strongly associated with leaf (red), rhizome (blue), and root (green). The node representing the OTU 1 was not associated to any community and is shown in purple. Black edges connect nodes at same community, and red edges connect nodes at different communities. The widths of the edges connecting OTUs to tissues are proportional to the total number of observations of each OTU in each tissue divided by the number of replicates. * = Statistically significant.

We sequenced approximately 200 bands detected by DGGE analysis, trying to cover all identified OTUs. However, we only managed to obtain 12 different bacterial sequences. Totally 33.3% of the sequences analyzed belonged to Bacteroidetes, while the rest (66.7%) belonged to the class Proteobacteria: 41.7% were affiliated to the α-subclass, 16.7% to the γ-subclass, and 8.3% to the δ-subclass. More specifically, 15.4% of the sequences belonged to the *Desulfovibrionaceae*, 15.4% to the *Flammeovirgaceae*, 15.4% to the *Rhodobacteracerae*, 15.4% were *Sphingobacteriaceae*, 15.4% non-identified Coral Black Band Disease isolates, 7.7% *Oceanimonaceae*, 7.7% *Rhizobiaceae*, and 7.7% were non-identified Sulfur-Oxidizing Symbionts. We identified three endophytic bacteria characteristic of leaf tissues, seven of rhizome tissues, and two of roots tissues. The closest relative sequences to our OTUs are listed in **Table A1** and the phylogenetic assignment is illustrated in **Figure 5**.

DISCUSSION

The results reported here provide a pioneering step toward the characterization of the endophytic bacterial community associated with tissues of a marine angiosperm, by both comparing DGGE band patterns and sequencing the main OTUs found. Our results show that endophytic bacteria are frequently present in tissues of *P. oceanica* in the Balearic Islands, as most samples analyzed (93.1% of roots, 81.4% of rhizomes, and 88.6% of leaves) carried endophytic bacteria. However, a more exhaustive survey using larger amounts of tissue and/or other techniques more sensitive to low amounts of DNA could yield even higher percentages of plants carrying bacterial endophytes.

Whereas our study appeared to yield a thorough inventory of OTUs in tissues of *P. oceanica*, the number of different OTUs identified in *P. oceanica* tissues, by DGGE analysis, appears relatively small, with 34 OTUs detected in *P. oceanica* tissues, which suggest that the endophytic microbiota must be highly specialized. The endophytic bacterial diversity reported here is similar to that found using comparable methodologies in other plants, such as rice (*Oryza sativa*), where 52 different endophytic OTUs were identified in a library of 192 clones. About 60% of endophytic OTUs detected on rice were Proteobacteria (Sun et al., 2008), similar to the percentage obtained for *P. oceanica* tissues. Similar numbers of epiphytic bacterial OTUs were obtained by DGGE in marine green *Ulvacean* algae where 34 sequences were

Table 2 | Indicator species test.

	Times of appearance in						
OTUs	**Roots**	**Rhizomes**	**Leaves**	**All**	**Cluster**	**Indicator value**	**Probability**
OTU_15	**1**	**4**	**12**	**17**	**Leaves**	**0.1344**	**0.001**
OTU_14	**7**	**5**	**17**	**29**	**Leaves**	**0.1612**	**0.004**
OTU_33	5	9	13	27	Leaves	0.0994	0.186
OTU_1	0	0	2	2	Leaves	0.0323	0.209
OTU_5	0	0	2	2	Leaves	0.0323	0.216
OTU_7	6	1	7	14	Leaves	0.0579	0.232
OTU_16	5	5	8	18	Leaves	0.0571	0.482
OTU_6	12	7	12	31	Leaves	0.0756	0.63
OTU_11	3	5	6	14	Leaves	0.0408	0.698
OTU_34	**0**	**8**	**0**	**8**	**Rhizomes**	**0.1404**	**0.001**
OTU_4	**15**	**21**	**6**	**42**	**Rhizomes**	**0.197**	**0.005**
OTU_19	**0**	**4**	**0**	**4**	**Rhizomes**	**0.0702**	**0.011**
OTU_10	**5**	**13**	**6**	**24**	**Rhizomes**	**0.1302**	**0.019**
OTU_12	**10**	**26**	**25**	**61**	**Rhizomes**	**0.2063**	**0.019**
OTU_31	0	2	0	2	Rhizomes	0.0351	0.074
OTU_23	1	3	0	4	Rhizomes	0.041	0.079
OTU_17	0	2	0	2	Rhizomes	0.0351	0.095
OTU_21	1	2	0	3	Rhizomes	0.0246	0.199
OTU_25	2	3	2	7	Rhizomes	0.0241	0.675
OTU_9	1	2	2	5	Rhizomes	0.015	0.875
OTU_26	**39**	**13**	**21**	**73**	**Roots**	**0.2949**	**0.001**
OTU_29	**24**	**2**	**0**	**26**	**Roots**	**0.3263**	**0.001**
OTU_30	**13**	**2**	**0**	**15**	**Roots**	**0.1643**	**0.001**
OTU_27	**14**	**0**	**8**	**22**	**Roots**	**0.1292**	**0.004**
OTU_2	**14**	**1**	**5**	**20**	**Roots**	**0.1422**	**0.005**
OTU_24	**17**	**8**	**1**	**26**	**Roots**	**0.1569**	**0.006**
OTU_8	7	2	2	11	Roots	0.0635	0.104
OTU_32	3	0	0	3	Roots	0.0448	0.136
OTU_18	11	3	7	21	Roots	0.0818	0.157
OTU_20	11	3	9	23	Roots	0.0745	0.288
OTU_22	5	0	4	9	Roots	0.04	0.289
OTU_13	18	12	11	41	Roots	0.1099	0.405
OTU_28	5	4	3	12	Roots	0.0288	0.921
OTU_3	1	0	0	1	Roots	0.0149	1

Number of times each OTU was found in each tissue class, and in total. Cluster indicates the group for which each OTU is a likely indicator, with the indicator value and the associated probability. Statistically significant indicator species shown in bold.

reported, most of which belonged to Proteobacteria with minor representation of Bacteroidetes (Tujula et al., 2010). However, the endophytic bacterial community characterized by DGGE analysis in potato (*Solanum tuberosum*) and maize (*Zea mays*) plants was less diverse with 11 OTUs for potato plants (Garbeva et al., 2001) and six different bacterial species, all identified as proteobacteria, for maize roots (Seghers et al., 2004). When compared with other marine organisms, the bacterial diversity described in *P. oceanica* is similar to that found in the marine sponge *Rhopaloides odorabile*, where 34 different bacterial sequences were obtained from a clone library of 70 clones obtained from of three samples (Webster et al., 2001).

Our estimates of bacterial endophyte richness are influenced by the choice of DGGE in our survey. Much higher numbers of OTUs could be expected from large cloning efforts or from the use of massively parallel sequencing techniques. Webster et al. (2010) found 2996 different bacterial OTUs in sponges using 454 tag sequencing while the same sponges only yielded 34 sequences out of a library of 70 clones (Webster et al., 2001). Similarly, 1178 clones obtained from only 14 coral samples yielded 430 distinct bacterial ribotypes of endo- and epibionts by clone library techniques (Rohwer et al., 2002). Thus, DGGE or cloning of a limited number of clones can only detect the highly abundant members of the bacterial community and it is possible that use of high-throughput sequencing techniques would result in the detection of a much larger number of low abundance endophytes. Therefore, the number reported here is a minimum estimate of the species richness of the bacterial endophytes of *P. oceanica*.

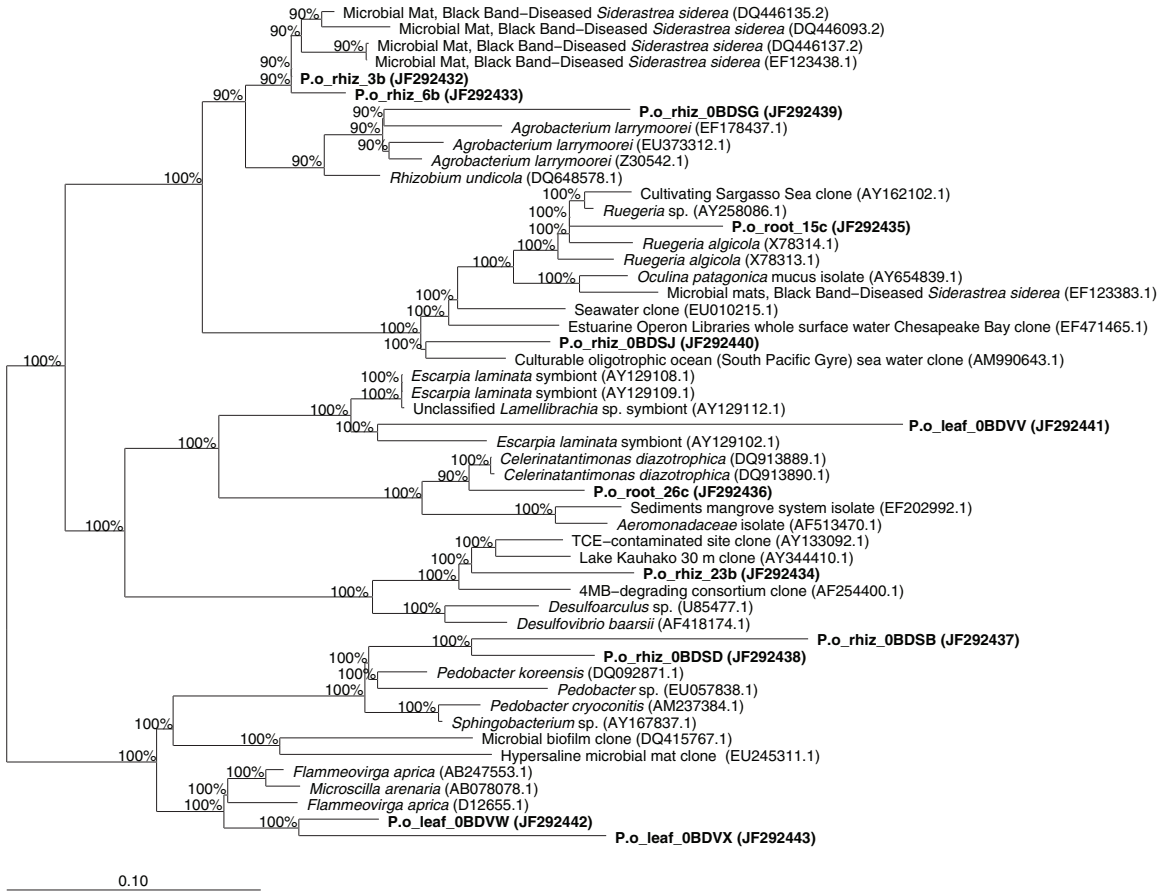

FIGURE 5 | Phylogenetic affiliation of the sequences obtained from the endophytic bacterial community of *P. oceanica*. Sequences obtained in this study are printed in bold. The values at each node represent the bootstrap values generated using the ARB parsimony interactive tool.

In contrast, most of the ribotypes reported by deep-sequencing studies are present in a very low abundance with only one or a few sequences out of several thousands or more (Webster et al., 2010). From a functional point of view, it is likely that those microbial types present in high abundance possess a biomass high-enough to contribute significantly to the metabolism of the plant, while the contribution of rare bacterial ribotypes is probably less significant. Thus, our minimal estimate of the highly abundant ribotypes obtained by DGGE analysis is likely to represent those bacterial endophytes having a more profound impact on the biology of *P. oceanica*.

The comparison of patterns in endophytic bacterial communities between tissues suggested that bacteria associated with roots differ from those associated with rhizomes and leaves, similar to what was found among rice tissues (García de Salamone et al., 2010). This was later confirmed by the ANOSIM test and the community analysis of bipartite networks (**Figure 4**). *P. oceanica* tissues experience a different range of physical and chemical environmental conditions due to their nature, such as light and oxygen concentration gradient and also toxic metabolites found mainly in sediment. The specific environment where each tissue is located can select for bacterial species that can survive. The presence of toxic metabolites such as sulfide that can intrude plant tissues (Frederiksen et al., 2007) can be conditioning the bacterial species that can survive and develop in each tissue compartment. Moreover, some OTUs were identified as indicator species in roots, rhizomes, and leaves of *P. oceanica*, confirming the existence of a distinct endophytic community in each tissue. Differences in endophytic bacterial community patterns in *P. oceanica* due to the type of tissue were small, suggesting the importance of the meadow from which the tissue was collected and therefore, the importance of environmental factors affecting each location. In each island we can find a broad range of anthropogenic perturbation, with some locations being highly impacted by bathing and boat traffic and some others more pristine receiving fewer visitors per year. The bacterial community composition appeared to be related to the geographical location of the sampled meadows (**Figure 3**). Those meadows located in Mallorca and Ibiza, the two islands subjected to higher pressure from tourism were most similar as compared to Cabrera and Formentera, the more pristine, less visited islands. This suggests that anthropogenic perturbation may have an impact on the bacterial communities inhabiting *P. oceanica* tissues.

The sequencing of the main OTUs detected by DGGE analysis allowed us to draw the first identification of the endophytic

bacterial community in *Posidonia oceanica* tissues. The main group represented is the Proteobacteria class comprising 66% of the OTUs, with the a-subclass being the majority group, as is characteristic for marine environments. The other main group is represented by Bacteroidetes, with many representatives found in marine environments. Most of the bacterial OTUs belonged to *Desulfovibrionaceae, Flammeovirgaceae, Rhodobacteracerae, Sphingobacteriaceae*, and Non-identified Coral Black Band Disease isolates. Less common groups were *Oceanimonaceae, Rhizobiaceae*, and Non-identified Sulfur-Oxidizing Symbionts.

The identification of bacteria similar in sequence to those found in diseased coral tissues opens a new and exciting research line, as there is no evidence, to our knowledge, of specific bacterial pathogens of seagrasses. However, demonstrating the pathogenicity of these organisms will require further research, involving the isolation of the potential causative agents and demonstrating that they fulfill Koch's postulates. Some of these bacteria found in diseased corals have been identified in association with macroalgae without relation to disease (**Table A1**) and others could be just opportunistic microbes degrading already damaged tissues. Some of the sequences were similar to those of sulfur-oxidizing symbionts (**Figure 5**). The presence of sulfur-oxidizing bacteria capable of oxidizing the sulfide to elemental sulfur would have an important role in detoxifying sediments because hydrogen sulfide produced as a consequence of organic matter decomposition is toxic for plants (Calleja et al., 2007; Garcias-Bonet et al., 2008). This would be particularly beneficial for *P. oceanica* survival in carbonate and iron poor sediments, characteristic of the Balearic coast and many other Mediterranean areas, where low iron available in these sediments prevents formation of iron sulfur compounds, and thus even small inputs of organic matter are able to enhance pore water hydrogen sulfide concentration (Holmer et al., 2003; Marbà et al., 2007).

Similarly, we identify bacteria similar to sequences found endophytically in other plants and related to *Rhizobiaceae* (**Figure 5**), with many species that are able to fix nitrogen in symbiosis with plants. The identification of bacteria related to well known nitrogen fixers is specially interesting because the Mediterranean sediments are known to be oligotrophic and the existence of bacteria with capabilities of shaping the nutrient conditions may have a beneficial role in the establishment, growth and survival of *P. oceanica* in this environment. This is particularly the case for bacteria belonging to the *Rhizobiaceae*, as *Agrobacterium* species are aerobic bacteria that can live free as well as some strains are responsible of tumor formation in terrestrial plants. In fact, there is a marine subdivision of *Agrobacterium* species (Uchino et al., 1997), although their role is still not clear. Moreover, PCR amplification of *nifH* genes from *P. oceanica* tissues confirmed the presence of diazotrophs (Garcias-Bonet et al., submitted).

In summary, this work is the first characterization of endophytic bacterial community in *Posidonia oceanica* tissues, suggesting the presence of specialized bacterial phylotypes in roots. The presence of bacterial endophytes in most of the samples analyzed indicates that these endophytes may be playing important roles in the physiology and survival of *P. oceanica* in the Mediterranean Sea. However, further research is needed to explain the different patterns observed across tissues and meadows. Moreover, this work represents the first identification of endophytic bacteria present in *P. oceanica* tissues. Some of the sequences were closely related to major groups of bacteria able to fix nitrogen, some others related to the sulfur cycle and finally a group of sequences had their closest known relatives among those found in diseased corals. It is not possible to infer whether or not the functional genes and capacities associated to the closest matching relatives will be present in our samples, due to the low similarity of some sequences to known cultured bacteria or even to environmental sequences. However, the fact that the closest matches are related to these three categories suggests that endophytic bacteria may play an important role in the health of *P. oceanica* by providing nitrogen and protecting the plants against the invasion of toxic sulfides. Moreover, the low sequence similarity to previously reported sequences in Genbank indicates that many of these sequences correspond to unknown bacteria, some of which could be specific to *P. oceanica* tissues. Subsequent research should include a search for functional genes involved in nitrogen fixation and the sulfur cycle and also a more detailed study on healthy vs. damaged tissues of *P. oceanica*, which could lead to the discovery of unknown bacterial pathogens of marine angiosperms.

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APPENDIX

Table A1 | Closest relatives of the sequenced OTUs.

Sequence ID (accession number)	Tissue/location	Length (bp)	Similarity (%)	Closest relative in BLAST (accession number)	Description	Author	Family/group in greengens
P.o_rhiz_3b (JF292432)	Rhizome/C.Sahona	494	99	Uncultured bacteria (EU181012)	Isolated from seagull fecal sample	Lu et al., 2008	Non-Identified Coral BBD isolates/α-proteobacteria
			99	Uncultured bacterium clone (GU118071)	Isolated from the coral *Acropora palmata*	Sunagawa et al., 2010	
			99	Uncultured bacterium clone (FJ202069)	Isolated from the coral *Montastraea faveolata* displaying signs of White Plague Disease type II	Sunagawa et al., 2009	
			99	Uncultured bacterium clone (EF123439)	Isolated from Black Band Diseased tissues of the coral *Siderastrea siderea*	Sekar et al., 2008	
			99	*Cohaesibacter* sp. (GQ200200)	Rhizobiales; Cohaesibacteraceae. Isolated from sediment of a seawater pond used for sea cucumber culture	Qu et al., 2011	
P.o_rhiz_6b (JF292433)	Rhizome/Figueral	433	97	Uncultured bacteria (EU181012)	Isolated from seagull fecal sample	Lu et al., 2008	Non-Identified Coral BBD isolates/ α-proteobacteria
			97	Uncultured bacterium clone (GU118071)	Isolated from the coral *Acropora palmata*	Sunagawa et al., 2010	
			97	Uncultured bacterium clone (FJ202069)	Isolated from the coral *Montastraea faveolata* displaying signs of White Plague Disease type II	Sunagawa et al., 2009	
			97	Uncultured bacterium clone (EF123439)	Isolated from Black Band Diseased tissues of the coral *Siderastrea siderea*	Sekar et al., 2008	
			97	*Cohaesibacter* sp. (GQ200200)	Rhizobiales; Cohaesibacteraceae. Isolated from sediment of a seawater pond used for sea cucumber culture	Qu et al., 2011	
P.o_rhiz_23b (JF292434)	Rhizome/Talamanca	518	92	Uncultured δ-proteobacteria (AY133092)	Isolated from TCE-contaminated site	Carrol and Zinder, Unpublished	*Desulfovibrionaceae*/ δ-proteobacteria
			92	Uncultured bacterium clone (GU118736)	Isolated from the coral *Montastraea franksi*	Sunagawa et al., 2010	
			91	Bacterium enrichment culture clone (HQ622261)	Polluted estuarine sediment	Abed et al., Unpublished	
			91	*Desulfarculus baarsii* (CP002085)	Desulfarculales; Desulfarculaceae; Desulfarculus	Lucas et al., Unpublished	

(Continued)

Table A1 | Continued

Sequence ID (accession number)	Tissue/location	Length (bp)	Similarity (%)	Closest relative in BLAST (accession number)	Description	Author	Family/group in greengens
P.o_root_15c (JF292435)	Root/C.Marmacen	471	96	*Roseovarius sp.* (HQ871860 + HQ871851)	Rhodobacterales; Rhodobacteraceae; Roseovarius	Jeanthon et al., Unpublished	*Rhodobacteraceae*/ α-proteobacteria
			96	*Pelagibaca sp.* (EU440959)	Rhodobacterales; Rhodobacteraceae; Pelagibaca	Yuan et al., Unpublished	
			96	Rhodobacterales bacterium (HQ537377 + HQ537273)	Isolated from 75 m depth on C-MORE BLOOMER cruise, Hawaii Ocean Time Series (HOT) station ALOHA"	Sher et al., Unpublished	
			96	Uncultured Rhodobacterales bacterium (GU474886)	Isolated from Hawaii Oceanographic Time-series study site ALOHA"	Rich et al., 2011	
			96	*Marinovum algicola* (FJ752526)	Rhodobacterales; Rhodobacteraceae; Marinovum	Pradella et al., 2010	
P.o_root_26c (JF292436)	Root/Magalluf	520	94	*Celerinatantimonas diazotrophica (DQ913889)*	Nitrogen Fixing Bacteria isolated from stuarine grasses *Spartina alterniflora* and *Juncus roemerianus*	Cramer et al., 2011	*Oceanimonaceae*/γ
			93	*Celeribacter arcticus* gen. nov., sp. nov. (FJ039852)	Alteromonadales. Halophilic denitrifying bacteria isolated from water brine in Siberian permafrost	Shcherbakova et al., Unpublished	
			92	Uncultured *Agarivorans sp.* (DQ647161)	Alteromonadales; Alteromonadaceae; Agarivorans	Dahle et al., 2008	
			92	*Agarivorans* sp. (GQ200591)	Alteromonadales; Alteromonadaceae; Agarivorans. Isolated from surface of seaweeds	Du et al., 2011	
P.o_rhiz_0BDSB (JF292437)	Rhizome/C.Torreta	391	91	Uncultured bacterium clone (GU946163)	Isolated from agricultural soil	Ros et al., Unpublished	*Sphingobacterriaceae*/ Bacteroidetes
			90	*Pedobacter sp.* (AM988948)	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Pedobacter. Isolated from lake water	Berg et al., 2009	
			90	*Pedobacter koreensis* (DQ092871)	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Pedobacter. Isolated from fresh water	Baik et al., 2007	

(Continued)

Table A1 | Continued

Sequence ID (accession number)	Tissue/location	Length (bp)	Similarity (%)	Closest relative in BLAST (accession number)	Description	Author	Family/group in greengens
P.o_rhiz_0BDSD (JF292438)	Rhizome/C.Torreta	412	96	Uncultured bacterium clone (HM125351)	Isolated from soils	Bissett, Unpublished	*Sphingobacteriaceae*/ Bacteroidetes
			95	Uncultured bacterium (AM158409)	Isolated from *Typha* rhizosphere in constructed wetlands	Saenz de Miera et al., Unpublished	
			95	Bacterium (FJ654260)	Isolated from soil	Kim et al., Unpublished	
			95	Uncultured bacterium clone (GU946163)	Isolated from agricultural soil	Ros et al., Unpublished	
			95	*Pedobacter sp.* (HM204919)	Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Pedobacter	Im, Unpublished	
P.o_rhiz_0BDSG (JF292439)	Rhizome/C.Torreta	368	92	Unidentified bacterium clone (EF606109)	Isolated from rhizosphere soil from former arable field sown with low seeds diversity	Kielak et al., 2008	*Rhizobiaceae*/ α-proteobacteria
			92	Uncultured bacterium clone (HM066499)	Isolated from environmental sample	Gray and Engel, Unpublished	
			92	Uncultured bacterium clone (AB583099)	Isolated from soybean leaf	Ikeda et al., 2011	
			92	*Agrobacetrium tumefaciens* (HQ003411)	Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium. Isolated from a lake	Sahay et al., Unpublished	
			92	*Agrobacetrium tumefaciens* (FJ999942)	Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium. Isolated from a plant, endophytic microbiota	Zheng and Feng, Unpublished	

(Continued)

Table A1 | Continued

Sequence ID (accession number)	Tissue/location	Length (bp)	Similarity (%)	Closest relative in BLAST (accession number)	Description	Author	Family/group in greengens
P.o_rhiz_0BDSJ (JF292440)	Rhizome/Porto Colom	404	97	*Nautella italica* (HQ908722)	Rhodobacterales; Rhodobacteraceae; Nautella. Bacteria associated with sponges	Feby and Nair, Unpublished	*Rhodobacteraceae*/ α-proteobacteria
			97	*Nautella* sp. (HQ188608)	Rhodobacterales; Rhodobacteraceae; Nautella. Isolated from surface seawater	Cho and Hwang, 2011	
			95	Uncultured bacterium clone (GU472165)	Isolated from BBD affected corals	Arotsker et al., Unpublished	
			97	*Ruegeria* sp. (GU176618)	Rhodobacterales; Rhodobacteraceae; Ruegeria. Isolated from surface of the red macroalgae, *Delisea pulchra*	Case and Kjelleberg, Unpublished	
			97	Rhodobacteraceae bacterium (FJ937900)	Rhodobacterales; Rhodobacteraceae. Isolated from *Gelliodes carnosa* (marine sponge)	Li et al., Unpublished	
			97	Uncultured bacterium clone (FJ202604)	Isolated from *Montastraea faveolata* kept in aquarium for 23 days	Sunagawa et al., 2009	
P.o_leaf_0BDVV (JF292441)	Leaf/Pujols	436	80	Uncultured gamma proteobacterium clone (DQ269096)	Isolated from surface of marine macro-alga *Delisea pulchra*	Longford et al., Unpublished	Sulfur-Oxidizing-Symbionts/ γ-proteobacteria
			80	Uncultured gamma proteobacterium clone (FJ205337)	Isolated from deep marine sediments	Dong and Shao, Unpublished	
			80	Uncultured bacterium clone (EU491600 + EU491489 + EU491463)	Isolated from seafloor lavas from the East Pacific Rise	Santelli et al., 2008	
			80	Uncultured gamma proteobacterium (AB611274)	Isolated from abdominal setae of galatheid crab (*Shinkaia crosnieri*) at the Hatoma Knoll in the Okinawa Trough	Yoshida-Takashima et al., Unpublished	
			80	Uncultured gamma proteobacterium clone (AY534017)	Isolated from oxic surface sediments of eastern Mediterranean Sea	Polymenakou et al., 2005	

(Continued)

Table A1 | Continued

Sequence ID (accession number)	Tissue/location	Length (bp)	Similarity (%)	Closest relative in BLAST (accession number)	Description	Author	Family/group in greengens
P.o_leaf_0BDVW (JF292442)	Leaf/Pujols	467	95	Sphingobacteriales bacterium (FJ952766)	Isolated from healthy tissue of coral *Montastrea annularis*	Rypien et al., 2010	*Flammeovirgaceae*/ Bacteroidetes
			95	*Flammeovirga aprica* (FJ917551)	Isolated from *Enteromorpha* (Green algae)	Zhang et al., Unpublished	
			95	*Flammeovirga sp.* (EF587968)	Isolated from natural subtidal biofilm	Huang et al., Unpublished	
			95	Uncultured bacterium clone (EF433127)	Isolated from *Favia* sp. mucus layer adjacent to Black Band mat	Barneah et al., 2007	
			95	*Flammeovirga sp.* (FN377813)	Bacteroidetes; Cytophagia; Cytophagales; Flammeovirgaceae; Flammeovirga. Isolated from a marine gastropod mollusk *Haliotis diversicolor*	Lu, Unpublished	
P.o_leaf_0BDVX (JF292443)	Leaf/Pujols	447	87	*Flammeovirga aprica* (FJ917551)	Isolated from *Enteromorpha* (Green algae)	Zhang et al., Unpublished	*Flammeovirgaceae*/ Bacteroidetes
			86	Sphingobacteriales bacterium (FJ952766)	Isolated from healthy tissue of coral *Montastrea annularis*	Rypien et al., 2010	
			86	*Flammeovirga sp.* (EF587968)	Isolated from natural subtidal biofilm	Huang et al., Unpublished	
			86	Uncultured bacterium clone (EF433134)	Isolated from *Favia* sp. mucus layer adjacent to Black Band mat	Barneah et al., 2007	
			86	*Flammeovirga aprica* (FN554611)	Isolated from a marine gastropod mollusk *Haliotis diversicolor*	Zhao, Unpublished	

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Phytoplankton chytridiomycosis: fungal parasites of phytoplankton and their imprints on the food web dynamics

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Parasitism is one of the earlier and common ecological interactions in the nature, occurring in almost all environments. Microbial parasites typically are characterized by their small size, short generation time, and high rates of reproduction, with simple life cycle occurring generally within a single host. They are diverse and ubiquitous in aquatic ecosystems, comprising viruses, prokaryotes, and eukaryotes. Recently, environmental 18S rDNA surveys of microbial eukaryotes have unveiled major infecting agents in pelagic systems, consisting primarily of the fungal order of Chytridiales (chytrids). Chytrids are considered the earlier branch of the Eumycetes and produce motile, flagellated zoospores, characterized by a small size (2–6 μm), and a single, posterior flagellum. The existence of these dispersal propagules includes chytrids within the so-called group of zoosporic fungi, which are particularly adapted to the plankton lifestyle where they infect a wide variety of hosts, including fishes, eggs, zooplankton, algae, and other aquatic fungi but primarily freshwater phytoplankton. Related ecological implications are huge because chytrids can killed their hosts, release substrates for microbial processes, and provide nutrient-rich particles as zoospores and short fragments of filamentous inedible hosts for the grazer food chain. Furthermore, based on the observation that phytoplankton chytridiomycosis preferentially impacts the larger size species, blooms of such species (e.g., filamentous cyanobacteria) may not totally represent trophic bottlenecks. Besides, chytrid epidemics represent an important driving factor in phytoplankton seasonal successions. In this review, I summarize the knowledge on the diversity, community structure, quantitative importance, and functional roles of fungal chytrids, primarily those who are parasites of phytoplankton, and infer the ecological implications and potentials for the food web dynamics and properties. I reach the conclusion that phytoplankton chytridiomycosis represents an important but as yet overlooked ecological driving force in aquatic food web dynamics and network organization.

Keywords: fungi, chytrids, microbial parasites, phytoplankton hosts, food webs, microbial ecology, aquatic ecosystems

INTRODUCTION

Parasitism is one of the earlier known and most common ecological interactions in nature (Cavalier-Smith, 1993), occurring in almost all environments (Lafferty et al., 2006). Parasites have received much more attention in terrestrial than in aquatic ecosystems (Kuris et al., 2008), where they represent a strong forcing factor for critical evolutionary and ecological processes, e.g., population dynamics, species successions, competition for resources, species diversification, and energy and gene flows (Hudson et al., 2006). Few attempts have been made to include parasites in the food web dynamics of aquatic systems (McCallum et al., 2004; Amundsen et al., 2009), with special emphasis on parasites of plants (Buschmann et al., 2001), invertebrates (Perkins, 1993), and vertebrates (Marcogliese, 2004).

Recent ecological and molecular surveys in pelagic environments have revealed a high occurrence of eukaryotic putative parasitoids, especially in the picoplanktonic size-fraction (López-García et al., 2001; Lefranc et al., 2005; Lefèvre et al., 2007, 2008), adding to the other typical parasitic entities such as viruses (Sime-Ngando and Colombet, 2009). Eukaryotic parasites known from 18S rDNA diversity surveys include fungal members of the Phylum Chytridiomycota as a major water-borne group, comprising both host-attached vegetative (i.e., sporangia) and free-swimming infective (i.e., flagellated zoospores) stages (Gleason and MacArthur, 2008). The Phylum Chytridiomycota (thereafter, chytrids) occupies the basal branch of the Kingdom Fungi and because associated members are small in size and lack conspicuous morphological features, chytrids are hardly distinguishable from many flagellated protists such as the sessile choanoflagellates or bicosoecids which are bacterivores (Lefèvre et al., 2007, 2008). Chytrids exhibit different trophic strategies (i.e., parasitism, saprotrophy) than these phagotrophic protists. In addition, chytrid propagules can represent key intermediates in the food chain (Gleason et al., 2009). Indeed, fungal zoospores have suitable

dimensions and represent a valuable food source for zooplankton. Similar to some protists (Desvilettes and Bec, 2009), fungal zoospores also contain essential fatty acids that might further upgrade the nutritional quality of food ingested by zooplankton such as *Daphnia* (Kagami et al., 2007a,b).

It is thus important to include eukaryotic parasites in the microbial ecology of aquatic environments. This is timely and will help to integrate "novel" ecological perspectives and extend the concept of parasitism and their functional potential to the aquatic food web dynamics (Gachon et al., 2010; Sime-Ngando and Niquil, 2011). This review focuses primarily on chytrid parasites of phytoplankton, and complements our recent review on eukaryotic microbial parasites in the plankton (Rasconi et al., 2011), by summarizing the knowledge on their diversity, community structure, quantitative importance, and ecological roles, and infer the ecological implications and potentials for the food web dynamics, properties, and overall topology.

CHYTRIDS: A DISREGARDED DIVERSITY WITHIN THE KINGDOM OF EUMYCOTA

The term Fungi globally embraces all organisms that belong to the kingdom Eumycota (i.e., the so-called true-fungi), while the term fungi also includes other microorganisms (i.e., fungus-like organisms) traditionally studied by mycologists such as members of Myxomycota (Amobozoa) also called slime molds, and of Oomycota (Heterokonts) also called water molds, as well as mushrooms and other molds (**Figure 1**). All these organisms share similar trophic strategies, namely saprophytism, parasitism, and other symbiotic associations, and can occur in the same ecosystem (reviewed in Jobard et al., 2010a; Wurzbacher et al., 2010, 2011). They constitute one of the last frontiers of the undiscovered biodiversity and the related functions that challenge aquatic microbial ecology today. The number of fungi present on earth was estimated to about 1.5 million species, from which approximately 97,000 have so far been identified, including about 300 species of anamorphic fungi and 300 species of Ascomycota but only few species of Basidiomycota and Chytridiomycota. Known fungal species correspond mostly to cultured specimens thriving in moist soils, lotic systems, mangroves, and wetlands, or to economically interesting pathogens of humans, plants, and animals. For example, *Batrachochytrium dendrobatidis*, the chytridiomycosis agent of one of the most deadly contemporary skin diseases that drive the decline of amphibian populations worldwide has been well studied (Voyles et al., 2009), leading to a great deal of publicity.

The kingdom Eumycota groups the following four divisions: Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota (James et al., 2006), but also include a particular group of microorganisms phylogenetically affiliated to the divisions Ascomycota and Basidiomycota (i.e., the so-called Deuteromycota), known as "fungi imperfecti" because only asexual forms (or anamorphs) have been observed during their life cycle. This group contains the particularly well studied members of Fungi in running waters, i.e., the hyphomycetes (Shearer et al., 2007). Zygomycota and Chytridiomycota do not emerge as monophyletic groups in recent phylogenetic analyses (James et al., 2000, 2006). Taxa traditionally placed in Zygomycota are distributed among Glomeromycota (**Figure 1**) and several subphyla *incertae sedis*, including Mucoromycotina, Entomophtoromycotina,

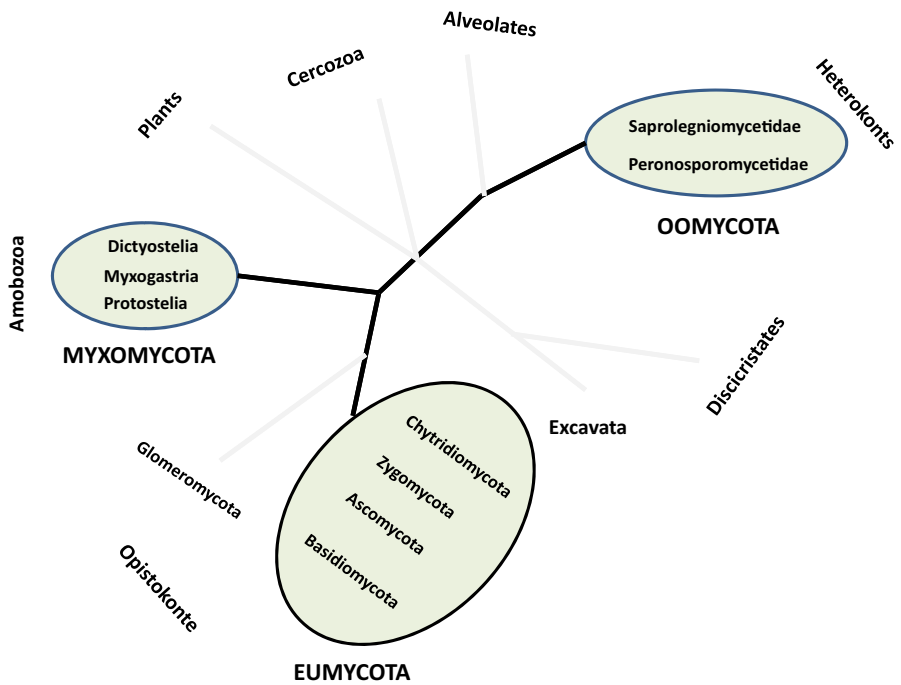

FIGURE 1 | Approximate position of true-Fungi (Eumycota) and fungus-like (Oomycota and Myxomycota) organisms in a schematic phylogenetic tree of eukaryotes. Modified from Jobard et al. (2010a).

Kickxellomycotina, and Zoopagomycotina. The Chytridiomycota is retained in a restricted sense, with Neocallimastigomycota and Blastocladiomycota representing segregate phyla of flagellated Fungi, also known as zoosporic Fungi. These zoosporic true-fungi (chytrids) were described according to modes of reproduction, thallus development and ecology and, most importantly, morphological characteristics of the thallus such as size, shape, and ornamentation of the sporangia, presence or absence of an operculum, and rhizoidal arrangement (Blackwell et al., 2006). Because they rely on free water phase for whole or part of their life cycle, chytrids are considered typical pelagic (i.e., floating) species.

ECOLOGICAL CONCEPTUALIZATION OF CHYTRID LIFE CYCLE AND ADAPTATION TO PELAGIC LIFESTYLE

Chytrid species have an interesting life cycle in the context of the pelagic realm where the two main stages (i.e., sporangium and zoospore) have different effects on the food web dynamics. Most members reproduce asexually by releasing zoospores with a single posteriorly directed whiplash flagellum (Sparrow, 1960; Barr, 2001). In a few species of the Neocallimastigales, zoospores are multiflagellate (Trinci et al., 1994) or in at least one species of the Blastocladiales (Hoffman et al., 2008) and one species in the Monoblepharidales (Ustinova et al., 2000), the spores lack flagella. The thallus can be either monocentric, polycentric, or filamentous (hyphal; Sparrow, 1960) and are able to grow either on top or within substrates. In the typical life cycle, a free-living zoospore encysts to the host and expands intracellularly as a tubular rhizoid, i.e., the nutrient conveying system for the formation of fruit bodies (i.e., the infective sporangium) from which propagules (i.e., motile zoospores) are released into the environment. The hosts of parasitic chytrids in aquatic systems are highly diverse, including both prokaryotic (i.e., cyanobacteria) and eukaryotic phytoplankton, protists, invertebrates (larvae of insects, rotifers, nematodes, crustacean such as copepods, ostracods, cladocera etc. . .), flowering plants or other fungi. Chytridiomycosis epidemics are known to produce massive amount of zoospores, now known as valuable food source for zooplankton (Kagami et al., 2007a,b). The two main development stages of chytrids thus highlight two overlooked ecological potentials in the food web dynamics: (i) parasitic predation of host populations, most of which are inedible (i.e., unexploited by grazers), and (ii) the subsequent trophic link via the release of suitable zoospore food for zooplankton. In addition, we have recently shown that chytrid parasitism of cells within the filaments of cyanobacteria during bloom events can result in a mechanical fragmentation of the inedible filaments into shorter-size edible filaments (Gerphagnon et al., submitted), thereby enhanced the contribution of fungal parasites to the bloom decline (**Figure 2**).

Chytrids are more commonly found growing in bodies of waters, primarily in freshwater environments, and in soils as well. In general, chytrids prefer environments with low osmotic potentials. Only three species (i.e., *Rhizophydium littoreum*, *Thalassochytrium gracilariopsis*, and *Chytridium polysiphoniae*) have been properly identified and partially characterized from brackish and marine ecosystems. These species are either facultative or obligate parasites of marine macroalgae and invertebrates. Also, some species of *Olpidium* and *Rhizophydium* are parasites of small marine green algae and diatoms (review in Gleason et al., 2011). Water-borne fungi have to face various difficulties and constraints characteristic of aquatic habitats, among which oxygen availability perhaps may be one of the most restrictive parameters. Except for Neocallimastigales in which all species are amitochondrial obligate anaerobes known from the rumen hindguts of herbivorous mammals where ambient temperatures approach 40°C, all chytrids are obligate aerobes and their growth rates are greatly inhibited by low dissolved oxygen concentrations (Gleason et al., 2008). Investigations of the tolerance to anoxia in the chytrids *Rhizophydium sphaerotheca* and *Phlyctochytrium punctatum* have revealed that these fungi are facultative anaerobes (Goldstein, 1960).

Adaptation to dispersal in running water is typical of some fungi which have developed spores with particular morphologies (e.g., large and multiradiate conidia of hyphomycetes) allowing successful attachment on substrates in flowing waters (Jobard et al., 2010a). Chytrids represent the sole true-fungi phylum with species producing flagellated cells in their life cycle. These temporary swimming life zoospores are particularly well adapted to dispersal in pelagic medium where chytrids represent the best studied group of fungi, primarily in lakes where they occur mainly as phytoplankton parasites (Ibelings et al., 2004; Rasconi et al., 2009, 2012). However, these studies remain limited, mainly because of methodological constraints and under sampling of pelagic ecosystems, where the occurrence of fungi is often considered as contamination from allochthonous watershed inputs (Goh and Hyde, 1996; Jobard et al., 2010a).

METHODOLOGICAL LIMITATIONS

Conventional methods for analysis of zoosporic fungi to date have mainly relied on direct observation and baiting techniques, with subsequent fungal identification using morphological characteristics (review in Marano et al., 2012). Earlier descriptions of chytrid parasites were based on microscopic observation of sporangia which exhibit morphological features that can allow approximate phenotypic identification of specimens in living samples or those preserved with Lugol's iodine (Rasconi et al., 2011). Such approaches have provided detailed descriptions of the morphological features using light or phase-contrast microscopy (Ingold, 1940; Canter, 1949, 1950, 1951). Subsequently, electron microscopy was used to describe different life stages, and a number of studies describing the ultrastructural cytology of fungal zoospores and spore differentiation are available (Rasconi et al., 2011). The chytrid *Blastocladiella* sp. was the first fungal model for detailed structural studies on sporogenesis (Lovett, 1963). The precise conformation of the flagellar rootlets and the spatial distribution of organelles in zoospores have been determined, providing the basis for chytrid taxonomy (review in Gleason and Lilje, 2009).

Ecological investigations of the dynamics of chytrid populations in natural environments have been improved more recently with epifluorescence microscopy. Several fluorochromes have been used, among which the chitin stain calcofluor white (CFW) penetrates infected host cells remarkably well and is more efficient for the observation and photomicrography of the complete rhizoidal system of parasites, which is an important criterion for chytrid identification (Rasconi et al., 2009; Sime-Ngando et al., 2012a).

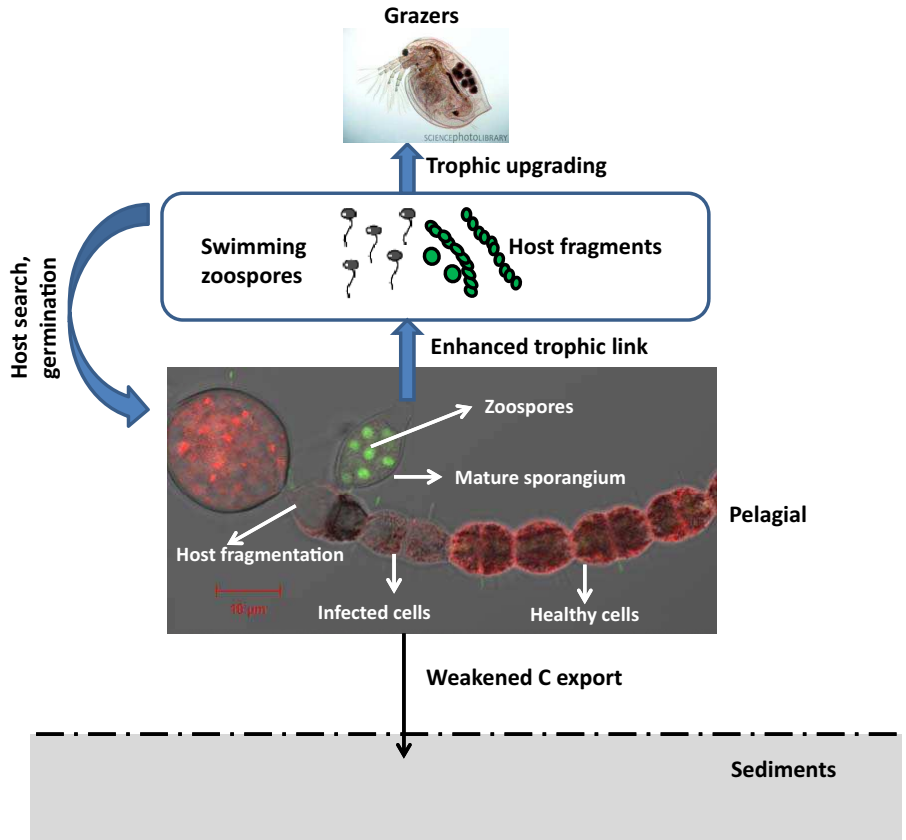

FIGURE 2 | Phytoplankton chytridiomycosis affects food web dynamics and properties by killing part of or the entire host filaments or free-living cells, and provide nutrient-rich particles as zoospores and short fragments of filamentous inedible hosts for the grazer (*Daphnia* in this case) food chain. Because chytrids preferentially attack larger size species such as the filamentous cyanobacterium *Anaebaena macroscopora* in this case, blooms of such species may not totally represent trophic bottleneck to be sequestered in the sediments. Part of their energy are retained in the pelagial and recycled through the trophic cascade. Source for Daphnia zooplankton micrograph is the science photo library: http://www.sciencephoto.com/media/367262/enlarge. The microphotograph of infected *A. macroscopra* filament with Cytox Green stained zoospores observed under confocal microscopy is a courtesy of Melanie Gerphagnon.

Molecular biology techniques have allowed a thorough reconstruction of chytrid phylogenies (James et al., 2006) and are increasingly providing more specific tools, primarily oligonucleotide probes, for the quantitative ecological study of aquatic parasites. Fluorescent *in situ* hybridization, amplified with horseradish peroxidase activation by fluorescent tyramide (also known as catalyzed reporter deposition, CARD FISH or TSA FISH), is a reliable approach to detect and count specific chytrid parasites, especially the zoospore stages that lack a chitinaceous wall, precluding any simple use of fluorochromes such as CFW (Sime-Ngando et al., 2012b). The clone-FISH approach, which was originally designed for prokaryotes, was recently adapted by Jobard et al. (2010b) to the assessment of zoosporic fungi in natural samples. Clone-FISH is based on the genetic modification of a clone of *Escherichia coli* by inserting plasmid vector containing the target 18S rDNA sequence. The main advantage of the clone-FISH method is that pure cultures which are necessary to validate the specificity and hybridization conditions of FISH or CARD FISH probing are not needed (Jobard et al., 2010b). Oligonucleotidic probes have also been used in quantitative real-time PCR (qPCR), which is an excellent tool for solving the limitations in the detection of less abundant and rare species (Lefèvre et al., 2010; Sime-Ngando and Jobard, 2012). Further application of PCR-base methods, primarily of next generation sequencing technologies, will not only advance our quantitative understanding of zoosporic fungal ecology and diversity, but also their function through the analysis of their genomes and gene expression (Monchy et al., 2011). Nevertheless, it is still necessary to complement these molecular-based approaches with cultivation-based methods in order to gain a fuller understanding of the ecological and physiological roles of zoosporic fungi (Marano et al., 2012).

The methodological difficulties are thus increasingly being overcome, and it is becoming evident that techniques from molecular biology are useful for the study of zoosporic fungi. The challenge of matching molecular sequences to microscopic phenotypes is also on the route to be tackled (Jobard et al., 2012; Monchy et al., 2012). Furthermore, to assess the functional impact of chytrid parasites on host populations, algorithms commonly

used by parasitologists such as the prevalence and the intensity of infection have been applied in ecological studies of chytridiomycosis (Rasconi et al., 2012). These parameters are derived from direct microscopy and are critical for assessing the community structure of parasites, interactions with hosts, and epidemiology, as well as the potential impact of fungal parasites on the food web dynamics. Understanding the environmental factors that induce epidemics can also be inferred this way (i.e., through empirical correlations) or by using epidemiological approaches such as the changes in incidence rates (i.e., the number of new cases of infections occurring during a given time) or the occurrence of epidemics (i.e., a widespread outbreak of an infection) within host populations (Fox, 2003).

SEASONAL DIVERSITY, ABUNDANCE, COMMUNITY STRUCTURE AND TROPHODYNAMICS OF CHYTRIDS

Studies on pelagic chytrids started in the British lakes (Cook, 1932), and different authors have provided descriptions of morphological characters (Reynolds, 1940; Canter and Lund, 1948, 1953; Pongratz, 1966; Canter, 1972). Quantitative assessment of the importance of parasitism indicated that infection of diatoms, desmids, and other green algae is fairly common in freshwaters (Canter and Lund, 1948, 1968). However, most studies on parasite dynamics in natural plankton assemblages are focused on hosts or limited to the investigations of one or two fungal populations (**Table 1**). This makes the generalization of observed patterns difficult, and the seasonal dynamics of chytrids at the community level remain largely unknown.

A recent extensive seasonal study in temperate freshwater lakes (Rasconi et al., 2012), based on CFW staining of infective sporangia and phenotypic identification, has identified up to 15 different chytrid species on diverse host populations, with specific biovolume ranging from 7 to 72 μm^3 sporangium^{-1} (**Table 1**). Seasonal abundances increased from 0.0005 to 0.4 $\times$ 10^6 sporangia l^{-1} in oligotrophic conditions to 0.0 to 32 $\times$ 10^6 sporangia l^{-1} in eutrophic conditions (**Table 1**). In both conditions, sporangium abundances peaked with the development of preferential diatom hosts in spring and cyanobacteria in autumn, the autumn peak being largely higher in eutro- than in oligotrophic conditions, when a monospecific bloom of *Anaebaena* sp. occurred. Quantitative data on the seasonal abundance of zoospores are lacking.

All the 15 species identified were monocentric (i.e., with one center of growth and development) and eucarpic (using part of the thallus for the fruit-body formation and with a specialized rhizoidal system), typical of the class *Chytridiomycetes*, and belonged to two orders: the *Rhizophidiales* which contained one genus (*Rhizophidium*), and the *Chytridiales* which contained three genera (*Chytridium*, *Zygorhizidium*, and *Rhizosiphon*). The species of *Rhizophidium* spp. infected a wide diversity of hosts, including both large size (e.g., the Chlorophyta *Staurastrum* spp. and the diatoms *Asterionella formosa*, *Synedra* spp. and *Fragilaria crotonensis*) and small size algae (e.g., the diatom *Cyclotella* spp. and the Chlorophyta *Chodatella ciliata* and *Ankistrodesmus convolutus*). The species *Chitridium* spp. infected the chlorophyte *Oocystis* sp., the diatom *F. crotonensis*, and the cyanobacterium *Microcystis* sp., while the species of *Zygorhizidium* infected the diatoms *Melosira* spp. The genus *Rhizosiphon* comprised two species that are specific parasites of vegetative cells and akinetes (*R. crassum*) and of akinetes alone (*R. akinetum*), which correspond to different niches offer by the filamentous cyanobacterium host *Anabaena macrospora* in productive lakes (Gerphagnon et al., submitted). Although almost all the chytrid species were observed from oligo- to eutrophic conditions, the seasonal fungal community composition was largely dominated by species of the genus *Rhizophidium* (90% of total sporangium abundance) in oligotrophic conditions, and of the genera *Rhizophidium* (56%), *Zygorizidium* (22%), *Chytridium* (19%), and *Rhizosiphon* (14%) in eutrophic conditions (Rasconi et al., 2012).

The community structure of natural chytrids is intimately linked to the availability of hosts (Ibelings et al., 2004). However, except the study by Rasconi et al. (2012) that has proposed a general empirical model on chytrid seasonality and trophodynamics (i.e., with their hosts) based on the theoretical PEG model of seasonal succession of planktonic events in freshwaters (Sommer et al., 1986), there is still no study assessing the fungal species successions in natural environments. This contrasts with the general hypotheses and patterns of plankton (primarily phytoplankton and zooplankton) successions and community structure, which are well described in temperate lakes (Sommer et al., 1986). This was recently revisited by considering a suite of overlooked ecological interactions that included parasitism (Sommer et al., 2012). These authors concluded that the effects of these "novel" interactions on plankton seasonal succession are limited in terms of seasonal biomass patterns but strong in terms of species replacements.

In the Rasconi and coauthor's model (Rasconi et al., 2012), during winter, the development and activities of both chytrid parasites and their phytoplanktonic hosts were at their lowest levels, because of low temperature, freezing, or ice-cover. From late winter on, the environmental conditions, primarily the increase in water temperature and in mixing-derived nutrient availability, favor the development of host communities, with the dominance of k-strategists (e.g., large diatoms) toward spring. As a consequence, the host – parasite contact probability increases, raising the chytrid infectivity and the production of large amount of zoospores. Enhanced infection prevalence then limits and provokes the decline of large diatoms, liberating niches for a diversified phytoplankton community of small size r-strategists. The abundance of chytrid sporangium reaches their lowest level, while the availability of food (i.e., small phytoplankton and fungal zoospores) favors the development of grazers and the establishment of a typical clear-water phase at the end of spring. During the summer months, favorable environmental conditions, together with a high grazing pressure, allow the development of a diversified and complex plankton community. Small edible hosts are inhibited by the grazing pressure, while the availability of large size hosts favors the proliferation of different chytrid species toward the end of the summer. From here, oligotrophic lakes significantly diverge from productive waters. In oligotrophic situations, autumnal overturn promotes species coexistence and phytoplankton diversity leads to the association of different species of chytrid parasites of chlorophytes and diatoms, but with general low infection prevalence due to a balanced host – parasite growth. In eutrophic lakes, nutrient conditions and persistent stratification favor the

Table 1 | Quantitative data on fungal parasites and parasitism of phytoplankton in temperate lake ecosystems.

Environment, country (trophy)	Method	Sampling period (depth, m)	Chytrid sporangia (10^6 l^{-1})	Sporangium biovolume (μm^3)	Prevalence of infection (% infected host cells)	Intensity of infection (sporangia host $cell^{-1}$)	Host (Chytrid)	Reference
Lake Pavin, France (O-M)	CFW staining and EM	Feb.–Dec. 2007 (Ze)	0.005–3.7	6.7–67.4	1.5–59.1	1–2.5	Phytoplankton communities (mixed)	Rasconi et al. (2012)
Lake Aydat, France (E)	CFW staining and EM	Feb.–Dec. 2007 (Ze)	0–3.4	8.7–72.4	0–98*	1–2	Phytoplankton communities (mixed)	Rasconi et al. (2012)
Lake Schöhsee, Germany (M)	Lugol staining and LM (Utermöhl)	Mar. 1987–May, 1989 (0–10)	ND	ND	0–>90** (≤1–10% of total host volume)	ND	Different species within Phytoplankton communities (mixed)	Holfeld (1998)
Lake Maarsseveen, The Netherlands (O-M)		1978–2010 (0–10)	ND	ND	0–90	ND	*Asterionella formosa (Rhizophydium planktonicum)*	Ibelings et al. (2004, 2011)
Lake Kinneret, Israel (O-M)	LM (Utermöhl)	Oct. 2000–Dec. 2003 (2 and 7)	ND	ND	0–83	ND	*Peridinium gatunense* (*Phlyctochytrium* sp.)	Alster and Zohary (2007)
Lake Schöhsee, Germany (M)	Lugol staining and LM (Utermöhl)	27 Jan.–7 Feb. 1989 (0–10)	ND	ND	∼10–80	∼0.1–1.8	*Stephanodiscus alpinus* (*Zygorhizidium* spp.)	Holfeld (2000)
Lake Suwa, Japan (E)	Dialysis tube cultures and LM	Nov. 1986–Nov. 1987 (0–4)	ND	ND	2–30	ND	*Asterionella formosa (Rhizophydium planktonicum or Zygorhizidium affluens)*	Kudoh and Takahashi (1990)
Shearwater, UK (E)		Several occasions between 1978 and 1981 (net samples)	ND	ND	0.2–1.4	Up to 4	Centric diatoms: *Cyclotella* spp., *Stephanodiscus hantzschii*, and *Melosira* spp. (mixed)	Sen (1988a)
Shearwater, UK (E)		10 epidemic periods between 1978 and 1980 (net samples)	ND	ND	15–90%	ND	*Microcystis aeruginosa* (*Rhizidium microcystidis*)	Sen (1988b)
Shearwater, UK (E)		Several occasions between 1978 and 1981 (net samples)	ND	ND	Up to 85%	ND	Several species of chlorophytes (mixed)	Sen (1988c)

**Recorded during a monospecific bloom of Anabaena flosaquae infected by Rhizosiphon crassum; **corresponds to the infection of Synedra acus infected by Zygorhizidium planktonicum.*

O, oligotrophic; M, mesotrophic; E, eutrophic or productive with recurrent cyanobacterial blooms; Ze, euphotic zone; CFW, calcofluor white (cf. Rasconi et al., 2009), EM, epifluorescence microscopy; LM, light microscopy; ND, not determined or not given explicitly.

bloom of filamentous cyanobacteria from the end of the summer period toward early autumn, with the development of a monospecific community of chytrids (i.e., *Rhizosiphon* spp.). The highest infection prevalence is noted, followed by the decline of cyanobacteria – chytrid system toward the late seasonal phase (for more details, see figure number 7 in Rasconi et al., 2012).

PHYTOPLANKTON CHYTRIDIOMYCOSIS AND THE INFLUENCE OF ENVIRONMENTAL FACTORS

A basis for study chytrid epidemics within phytoplankton communities was recently provided for freshwater lakes where, in contrast to sporangium abundance and biomass that increased from oligo- to eutrophic conditions, the prevalence of infection is quite similar in both conditions, averaging about 20% (Rasconi et al., 2012). The highest prevalence (98%) was noted for the autumn bloom of a filamentous cyanobacterium (*A. macospora*) facing the parasite *Rhizosiphon crassum* in a productive lake. The host species composition and their size appeared as critical for chytrid infectivity, the larger hosts being more vulnerable, including pennate diatoms, desmids, and filamentous cyanobacteria. Such host species are apparently easier to hit because their size naturally increase the host – parasite contact rates, and are expected to excrete more attracting substances known to favor the zoospores searching of suitable hosts (Canter and Jaworski, 1981). Larger algae also contain more resources for the diet of parasites, and this is the common explanation of why algal species with larger cell size can be heavily infected, even at lower population density (Lund, 1957; Holfeld, 1998). On the other hand, the prevalence of infection was also shown to be correlated with total phosphorus, which may be related to the productivity of the milieu that offers good substrate conditions for the growth of parasite-host systems (Rasconi et al., 2012). The abundances but also the cell volumes of hosts thus seem important features in determining the amplitude of chytrid epidemics within natural phytoplankton. These parameters also appeared to be related with the tolerance threshold of infection, i.e., the critical prevalence or the level of prevalence from which the standing stock of phytoplankton starts to decline (Bruning et al., 1992). At low host abundance, the critical infection prevalence is generally lower that 20%, but increases with increasing host abundance. This is probably one of the mechanisms from which parasites regulate host populations.

Diverse environmental conditions, including temperature, turbulence, light, nutrient concentrations, and biotic factors such as predation but primarily the host availability, can influence the growth rate of fungal parasites (Canter and Jaworski, 1981; Bruning and Ringelberg, 1987; Bruning, 1991; Kagami et al., 2004). The primary factor determining the absence or the presence of a particular parasite in the environment is the availability of suitable hosts (Ibelings et al., 2004). The host population density is frequently considered an important factor in the ecology of parasites. Indeed, irrespective of temperature and light conditions, a minimal threshold value of the host density is required for the occurrence of epidemics (Bruning, 1991). Several studies have reported that parasites seem to grow better on healthy individuals within actively growing host populations (Canter and Lund, 1948; Van Donk and Ringelberg, 1983). However, this is far from being a generalization because evidence was also provided that epidemics in natural phytoplankton populations arise more easily when growth conditions for hosts are worst (Reynolds, 1984). Under such conditions, the growth rate of the algae would be relatively slow, contrasting with their fast-growing parasites. Rasconi et al. (2012) hypothesized that there are two different phases in the parasite – host trophodynamic: a synchronous growth phase of both chytrids and algae when high availability of hosts favored the encounter between parasites and newly produced sensible host cells, followed by a second phase corresponding to the decline of host populations due to the infection, characterized by an increase in the infection prevalence.

ECOLOGICAL IMPLICATIONS OF PHYTOPLANKTON CHYTRIDIOMYCOSIS

Based on the study by Rasconi et al. (2012), the prevalences of infection (% of infected host cells) typically average around 20%, with no significant variation with the trophic status of freshwater temperate lakes. These values increase to reach about 100% when monospecific blooms of infected hosts occurred in natural conditions or when specific chytrid-host systems are targeted in controlled conditions (**Table 1**). Chytrid infection commonly leads to the death of their host cells (Canter and Lund, 1951; Sen, 1988b,c; Kudoh and Takahashi, 1990; Bruning et al., 1992; Holfeld, 1998, 2000; Ibelings et al., 2004, 2011) and this is enhanced by the intensity of infection (number of parasites per host cell) which can largely exceed 1 (**Table 1**). Empty sporangia are currently found attached on dead phytoplankton cells (Rasconi et al., 2012), which is suggestive of the lethal issue of chytrid infection. There are several evidences that parasitism inhibits the development of sensible species, and particular attention has been paid to the occurrence of fungi on diatoms, and to the effects of parasitism on their seasonal distributions (Canter and Lund, 1948; Van Donk and Ringelberg, 1983). For example, in the oligotrophic Lake Pavin (France), the spring development of the diatoms *Asterionella* and *Synedra* was found to be inhibited by the chytrid *Rhizophidium planktonicum*. In productive Lake Aydat (France) another diatom, *Fragilaria*, became abundant but the proliferation of their parasites, *Rhizophidium fragilariae*, interrupted their development (Rasconi et al., 2012).

In natural phytoplankton community, the parasitized populations are often replaced by others with similar ecologically requirements, which can render unchanged the standing stocks of phytoplankton hosts in the ecosystem, with no visible obvious damage to the total community (Reynolds, 1940). However, chytrids seem to preferentially infect large and less edible phytoplankton species, as discussed previously.

Some examples provided in the literature suggest that large infected diatoms such as *Asterionella* sp. and *Fragilaria* sp. (mean length 50 and 70 mm, respectively) can be replaced by small centric diatoms such as *Stephanodiscus* spp. (10 mm; Van Donk and Ringelberg, 1983; Sommer, 1987). This implies that the development of large species is inhibited by infection, while smaller algae proliferate. Active parasitism may thus act on the host standing stock in a continuum from no change to significant changes but in all cases will affect the phytoplankton community structure. In the context of phytoplankton seasonal dynamics and species successions, this can have profound ecological implications (Van

Donk, 1989). For example, due to chytrid parasites, the phytoplankton community can shift from a mature stage of development typically dominated by large, k-strategist species toward a pioneer stage of succession that favors the development of small, r-strategist species. In addition, by controlling the phytoplankton dynamics, chytrids can significantly affect the primary production of aquatic systems, as suggested by a negative correlation between the primary production and the per sporangium biovolume of chytrids in lakes (Rasconi et al., 2012).

More importantly, phytoplankton chytridiomycosis produce massive amount of propagules, i.e., zoospores. Because chytrids are small in size and lack conspicuous morphological features, a situation that makes them hardly distinguishable from many flagellated protists such as the sessile choanoflagellates or bicosoecids, their functional roles, primarily as saprotrophs or parasites, remain most of the time cryptic in classical microscopy studies (Lefèvre et al., 2007, 2008; Sime-Ngando et al., 2011). Previously, the modes of nutrition for all heterotrophic flagellates in the plankton were thought to be restricted to bacterivory, but zoosporic fungi are not bacterivores (i.e., bacterial feeders). It is now clearly evident that not all heterotrophic flagellates thriving in pelagic systems are either protists or bacterivores as previously thought, and that parasitism and saprophytism from fungal flagellates might represent important potential functions in these ecosystems. Fungal zoospores are valuable food sources for zooplankton because cytoplasm of chytrids contains storage carbohydrates such as glycogen, storage proteins, a wide range of fatty acids, phospholipids, sterols, and other lipids (Gleason et al., 2009; Sime-Ngando, in press). When chytrids reproduce, most of the cytoplasm is converted into zoospores which swim away to colonize new substrates or infect new hosts. Lipids are considered to be high energy compounds, some of which are important for energy storage. Indeed, lipids are present mainly in the form of endogenous reserves, often as membrane bound vesicles called lipoid globules which can easily be seen in the cytoplasm of fungal zoospores with both the light and electron microscopes (Gleason and Lilje, 2009; Sime-Ngando, in press). The size and numbers of lipoid globules within zoospores varied and their ultrastructure is complex. The chemical composition of lipids, including both fatty acids and sterols, has been characterized in a number of genera of zoosporic fungi. These endogenous reserves are consumed during the motile phase of the zoospores. They presumably provide energy for the movement of flagella during the motile phase which can last for up to several hours, as well as for the attachment and germination of zoospores on the appropriate substrates or hosts (**Figure 2**). Besides, many zoosporic fungi can grow in the laboratory on minimal synthetic media containing one carbon source such as cellulose, xylan, starch, or chitin along with salts containing nitrate, sulfate, and phosphate (Gleason et al., 2008; Sime-Ngando, in press). This establishes fungi as potential competitors of bacteria and primary producers for essential minerals (**Figure 2**).

There are other significant functions for the high energy compounds found in fungal zoospores, especially as food resources for zooplankton and probably for many other consumers in aquatic ecosystems. Fungal spores and hyphae in general are known to be eaten by a large number of different consumers in both aquatic and soil ecosystems, including a variety of mycophagous protozoa such as amebae and flagellates, detritivores, grazers such as filter feeding zooplankton, and benthic suspension feeders (Gleason et al., 2009; Sime-Ngando, in press). Since most of these consumers do not discriminate between food resources except by size, we would expect zoospores as well as hyphae and non-motile spores to be eaten by many of these consumers, although published records are lacking. Fungal zoospores are well within the range of a good particle size for zooplankton feeding behavior and consequently, when fed upon, transfer matter to higher trophic levels in the food chain. For example, zoospores are efficiently grazed by crustacean zooplankton such as Daphnia spp. (Kagami et al., 2007a,b, 2011; Sime-Ngando, in press), before they grow into a mature thallus (i.e., body). Thus zoospores may provide organic compounds containing nitrogen, phosphorus and sulfur, mineral ions, and vitamins to grazing zooplankton (**Figure 2**).

More interestingly, zoospores are a particularly good food source because of their nutritional qualities. Presumably many consumers must obtain at least some essential nutrients from their food sources because these compounds cannot be produced *de novo*. One example is found in the cladoceran *Daphnia*. Recent research has shown that zoospores of the parasitic chytrid, *Zygorhizidium*, are quite rich in polyunsaturated fatty acids (PUFAs) and cholesterols, which are essential nutrients for the growth of *Daphnia* (Kagami et al., 2007a,b). These zoospores are found to facilitate the trophic transfer from the inedible large diatom hosts, *Asterionella* sp., and the growth of *Daphnia*. PUFAs and cholesterol are known to promote growth and reproduction in crustacea. This phenomenon, known as the "trophic upgrading concept" (Sime-Ngando et al., 2011; Sime-Ngando, in press), is of significant importance in the aquatic food webs because it highlights not only the quantity but also the quality of the matter being transferred via fungal zoospores (**Figure 2**).

IMPACT OF PHYTOPLANKTON CHYTRIDIOMYCOSIS ON THE FOOD WEB PROPERTIES

Given that food webs are central to ecological concepts (Pascual and Dunne, 2006), it is important to establish the role of parasites in the structure and function of food webs. In theory, parasites can have a variety of effects. Lafferty et al. (2006, 2008) suggested that parasites affect food web properties and topology since they double connectance (defined as the number of observed links divided by the number of possible links) and quadruple the number of links. Others have postulated that parasites drive an increase in species richness, trophic levels, and trophic chain length of the food web (Huxham et al., 1995; Thompson et al., 2005). These properties may stabilize community structure (Hudson et al., 2006). However, the potential effects of parasites on food web stability is a complex and unresolved issue since the concept of stability is the center of a perhaps infinite debate in community ecology (Elton, 1958; May, 1972; Pimm, 1984; McCann, 2000; Hosack et al., 2009; O'Gorman and Emmerson, 2009). Based on the ideas of May (1973), parasites should lead to a destabilized trophic network because they increase species diversity and the connectance. In addition, adding parasites to food webs extends the length of trophic chains, which can decrease food web stability (Williams and Martinez, 2004). However, the addition of long loops of weak

interactions, which may be a characteristic of parasites with complex life cycles, might offset the destabilizing effects of increased connectance (Neutel et al., 2002).

To investigate ecosystem properties and ecological theories, the application of mathematical tools, such as models, is useful and allows trophic network representation through carbon flows (**Figure 3**). In the absence of quantification of the flows induced by fungal parasites of phytoplankton, we recently simulate their potential role in the plankton food web of the Lake Biwa, Japan (Niquil et al., 2011). The presence of this indirect pathway channeling microphytoplankton production to the consumers via the fungi leads to an enhancement of the trophic efficiency index, and a decrease of the ratio detritivory: herbivory. The results suggested that the food web relies less on the consumption of detritus, and that the transfer of carbon to higher trophic levels is higher than estimated without taking into account the parasites. Due to the lack of data quantifying carbon transfer through parasitism in pelagic ecosystems, no attempt was made to build model based on field estimated flows. Thus, the roles and ecological implications of chytrid infections of phytoplankton remain to be fully explored for aquatic microbial food webs.

As a first attempt, we recently provided such exercise for the first time, by adding parasitic chytrids of phytoplankton as an individualized compartment in a well studied pelagic food web and quantify their impact on matter flow through a trophic network (Grami et al., 2011). To describe the food web, models representative of carbon flows were built, including chytrid parasitism and the amount of primary production channeled in food web via chytrid infection. Carbon flows between the complete food web including parasitic chytrids (MWC, Model with Chytrids) were compared to the same model that did not consider the presence of chytrids and the resulting flows (MWOC, Model without Chytrid), as traditionally done in previous plankton food web analysis (e.g., Niquil et al., 2006). MWC and MWOC models were constructed on the basis of the same data set corresponding to the spring bloom period in Lake Pavin (i.e., March–June 2007). These models were built using the Linear Inverse Modeling procedure (LIM, Vézina and Platt, 1988) recently modified into the LIM-Monte Carlo Markov Chain (LIM-MCMC; Van den Meersche et al., 2009). This method allow reconstruction of missing flow values and alleviates the problem of under sampling using the principle of conservation of mass, i.e., the quantity of carbon coming into each compartment considered as equal to the amount leaving it (Vézina and Platt, 1988). Thanks to recent development of the inverse analysis into LIM – MCMC, a probability density function covering the range of possible values was generated for each flow. The results of this exercise are summarized in **Figure 3** where the inclusion of the two life stages of chytrid parasites of microphytoplankton (>20 μm) increases the number of compartments and flows. These parasites were able to short-circuit about 20% of the gross primary production, of which 15% is transferred to grazers with high throughput (**Figure 3**).

In addition, for each calculated set of flows generated by the Linear Inverse Modeling procedure, there is a set of calculated indices which allows application of statistical tests. The flows obtained from the models were used for calculations of Ecological Network Analysis indices that characterize the structure of the food web, and help reveal emergent properties (Ulanowicz, 1986, 1997, 2003; Ulanowicz et al., 2009). The use of ecological indices moreover, allows an indirect evaluation of the effects of network properties on the stability of the ecosystem, as several authors have proposed theoretical links between structural properties and local

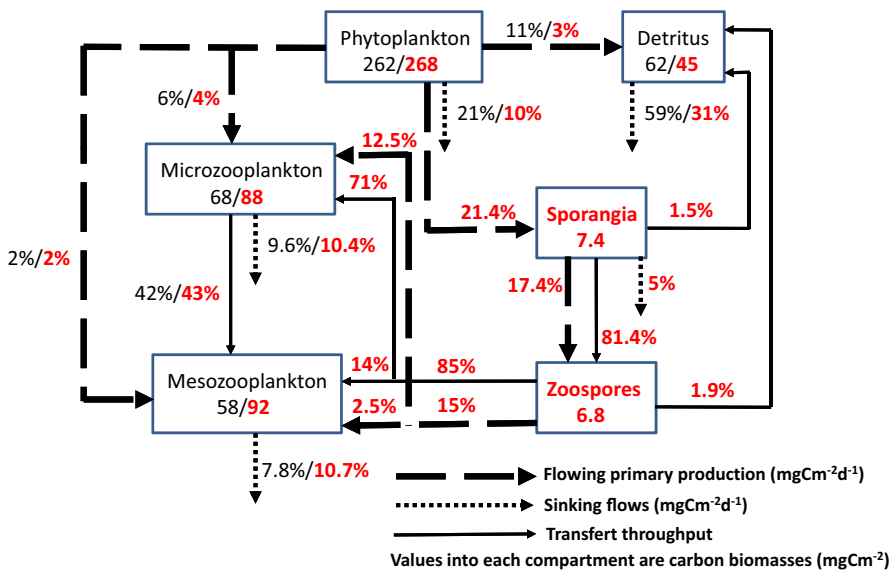

FIGURE 3 | Impact of parasitic chytrids on the microbial loop: flowing and sinking carbon from the gross primary production of phytoplankton (>20 μm) during the spring diatom bloom in the oligo-mesotrophic Lake Pavin, France. The effects of infective fungal sporangia and their propagules (zoospores) are highlighted in red color. The diagram corresponds to steady state models of the euphotic zone of the lake generated from a linear inverse modeling analysis. For more details, see the main text. Modified from Grami et al. (2011).

stability (cf. Ulanowicz, 2003). On these bases, the model results support recent theories on the probable impact of parasites on food web function. In the lake, during spring, when "inedible" algae (unexploited by planktonic herbivores) were the dominant primary producers, the epidemic growth of chytrid parasites significantly reduced the sedimentation loss of algal carbon from 21 to 10% of gross primary production (**Figure 3**). Furthermore, from the review of some theories about the potential influence of parasites on ecological network properties, we argue that parasitism contributes to longer carbon path lengths, higher levels of activity and specialization, and lower recycling. We then conclude that considering the "structural asymmetry" hypothesis as a stabilizing pattern, chytrids should contribute to the stability of aquatic food webs (Grami et al., 2011).

CONCLUSION

Chytrid symbionts and the related trophic modes, primarily parasitism, are omnipresent in aquatic ecosystems, including marine habitats (Gleason et al., 2011). In these ecosystems, they are diversified, with different taxa featured by different biological characteristics and requirements that determine their distributions in relation to environmental parameters but primarily to the seasonal dynamics of their phytoplankton hosts. The host abundance but also the host cell size and biomass likely establish the threshold limit for the critical prevalence of infection and the related decline in host communities. Related ecological implications are huge, because chytrid parasites can kill their hosts, release substrates for microbial processes, and provide nutrient-rich particles as zoospores and short fragments of filamentous hosts for the grazer food chain. This implies that cyanobacterial blooms, and other large size inedible phytoplankton blooms as well, may not totally represent trophic bottlenecks. Based on the observation that phytoplankton fungal parasitism preferentially impacts the larger size species (i.e., characteristics of climax populations), chytrid epidemics represent an important driving factor in phytoplankton successions and maturation, in addition to the sole seasonal forcing. The activity of chytrid parasites of phytoplankton thus represents an important but as yet overlooked ecological driving force in aquatic food web dynamics. In addition to being able to resist adverse conditions and use different sources of carbon and nutrients, chytrid parasites can indeed affect the plankton food web functions and ecosystem properties and topology, such as stability and trophic transfer efficiency. We are perhaps approaching a new paradigm-shift point in the development of aquatic microbial ecology (Sime-Ngando and Niquil, 2011).

However, the available study on phytoplankton chytridiomycosis remain restricted to a few temperate lakes, and extensive studies in the world's aquatic ecosystems, at wide geographical and time scales, are needed. Besides, the identification of chytrid species based on phenotypic features requires time and experience, and chytrid diversity provided this way probably is an underestimate. In this context, the increasing development of molecular tools is promising and will, in the near future, improves the linkage of cell identity and function, which is critical for an accurate assessment that includes microbial parasites in the carbon flows, and the related biogeochemical cycling in aquatic ecosystems (Jobard et al., 2012). Furthermore, parasitic lifestyle is generally highly subtle and can, for example, control competition by dominant species for resources, thereby promoting species coexistence and diversity. Parasites can also form long-lived associations with hosts, reducing their fitness for survival, or allowing infected hosts to remain strong competitors, although few models exist for microbial fungus-host systems.

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The parasitic dinoflagellates *Blastodinium* spp. inhabiting the gut of marine, planktonic copepods: morphology, ecology, and unrecognized species diversity

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Blastodinium is a genus of dinoflagellates that live as parasites in the gut of marine, planktonic copepods in the World's oceans and coastal waters. The taxonomy, phylogeny, and physiology of the genus have only been explored to a limited degree and, based on recent investigations, we hypothesize that the morphological and genetic diversity within this genus may be considerably larger than presently recognized. To address these issues, we obtained 18S rDNA and ITS gene sequences for *Blastodinium* specimens of different geographical origins, including representatives of the type species. This genetic information was in some cases complemented with new morphological, ultrastructural, physiological, and ecological data. Because most current knowledge about *Blastodinium* and its effects on copepod hosts stem from publications more than half a century old, we here summarize and discuss the existing knowledge in relation to the new data generated. Most *Blastodinium* species possess functional chloroplasts, but the parasitic stage, the trophocyte, has etioplasts and probably a limited photosynthetic activity. Sporocytes and swarmer cells have well-developed plastids and plausibly acquire part of their organic carbon needs through photosynthesis. A few species are nearly colorless with no functional chloroplasts. The photosynthetic species are almost exclusively found in warm, oligotrophic waters, indicating a life strategy that may benefit from copepods as microhabitats for acquiring nutrients in a nutrient-limited environment. As reported in the literature, monophyly of the genus is moderately supported, but the three main groups proposed by Chatton in 1920 are consistent with molecular data. However, we demonstrate an important genetic diversity within the genus and provide evidences for new groups and the presence of cryptic species. Finally, we discuss the current knowledge on the occurrence of *Blastodinium* spp. and their potential impact on natural copepod populations.

Keywords: *Blastodinium*, copepod, parasite, symbiont, plankton, ultrastructure, phylogeny

INTRODUCTION

The typical dinoflagellate is a motile, bi-flagellated protist, and species of the group may be found in both marine and fresh waters. Roughly half of all dinoflagellates are photosynthetic and half are heterotrophic (Gaines and Elbrächter, 1987). In addition, it has been estimated that approximately 7% of the dinoflagellates have parasitic life strategies (Drebes, 1984), infecting other protists, cnidarians, crustaceans, fishes, etc. (Coats, 1999) and some of these parasites can be severe pathogens for wild and farmed aquatic organisms. A key morphological feature of the dinoflagellates is their nucleus, the dinokaryon, which differs from the typical eukaryote nucleus by having permanent condensed chromosomes and by lacking histones. However, some of the parasitic dinoflagellates deviate from this typical morphology. In the dinoflagellate order Syndiniales a dinokaryon is never present, and those species that have traditionally been referred to the order Blastodiniales are believed to have a dinokaryon only in some parts of their live cycles. *Blastodinium* is a genus of dinoflagellates that appears atypical in several aspects. The parasitic stage of *Blastodinium* exists exclusively inside the gut of marine free-living copepods, where it occupies the lumen of the intestinal tract (**Figure 1A**). This parasitic stage is multicellular. It consists of several hundred non-flagellated cells, and can reach a length of more than 1 mm. The dispersal stage, the dinospore, of *Blastodinium* has the morphology of a typical dinoflagellate.

The greater part of our existing knowledge on *Blastodinium* leads back to the immense work done by the French protozoologist Édouard Chatton (Soyer-Gobillard, 2006) in the first half of the twentieth century, and his 1920 monograph (Chatton, 1920) on the parasitic dinoflagellates is still the primary landmark of several dinoflagellate genera and in particular of the genus *Blastodinium*. In addition to discovering and describing the

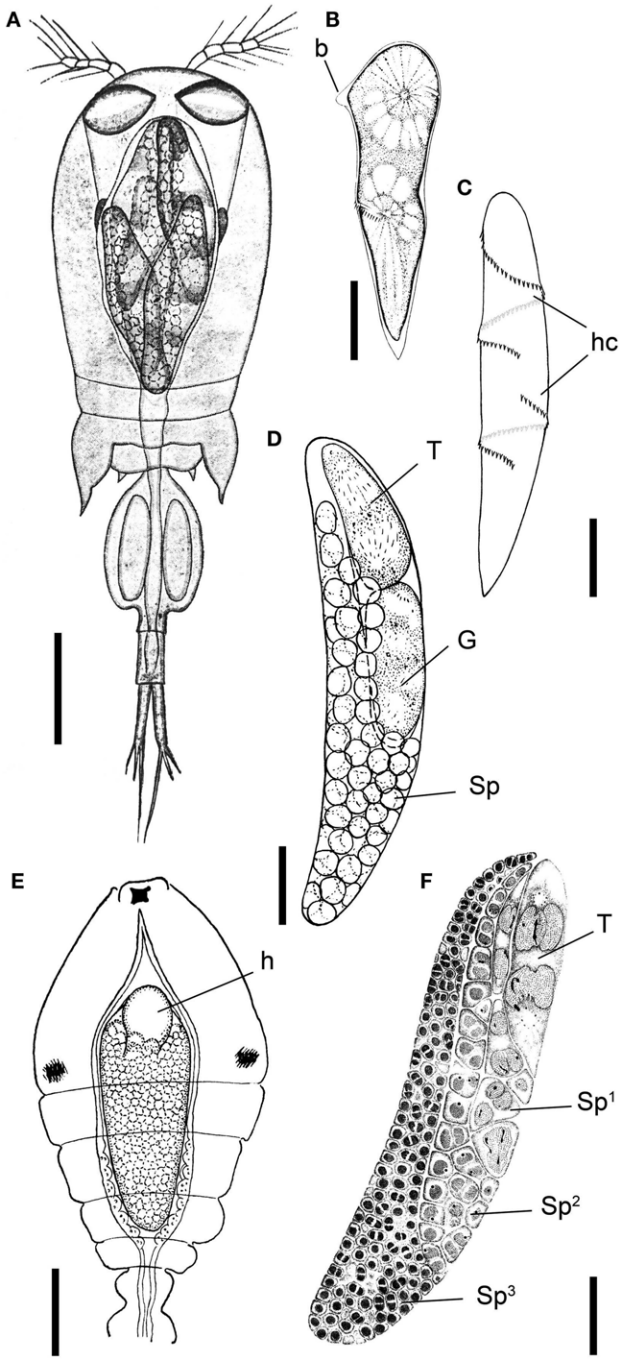

FIGURE 1 | *Blastodinium* spp. (A) Several *B. navicula* inside the gut of the copepod *Corycaeus giesbrechti*. **(B)** A young trophocyte of *B. pruvoti* with anterior beak (b). **(C)** Early stage *B. spinulosum* with helicoidal crests (hc). **(D)** *B. pruvoti* showing trophycyte (T), gonocyte (G), and sporocytes (Sp). **(E)** *B. oviforme* with hilum (h) in *Oithona nana*. **(F)** *B. pruvoti* with three progressive layers of sporocytes (Sp^1–Sp^3). Adapted from Chatton (1920). **(A,E)** Scale bars = 100 μm. **(B–F)** Scale bars = 50 μm.

genus in 1906, Chatton also described most known *Blastodinium* species in succeeding publications (**Table 1**). Since then, only three new species of *Blastodinium* have been described (Sewell, 1951; Skovgaard and Salomonsen, 2009), but recent investigations have shown that the genetic diversity within the genus is high, suggesting a larger number of unknown species (Coats et al., 2008; Skovgaard and Salomonsen, 2009; Alves-de-Souza et al., 2011). Investigations of the biology and impact of *Blastodinium* spp. are remarkably rare considering the large amount of research that is continuously being carried out on the biology and ecology of marine copepods. In this paper we update the current knowledge on the biology, phylogeny, and morphology of *Blastodinium* spp. and the current reports of distribution and host range of *Blastodinium* spp. are summarized. The established data is supplemented with new observations on morphology, ultrastructure, molecular phylogeny, and photosynthetic potential of *Blastodinium* spp. Due to the photosynthetic capability of *Blastodinium* and the apparently weak pathology associated with the infection, it may be a matter of definition whether members of the genus should be categorized as parasites or symbionts. We here adopt the term parasite because some harmful effect has been documented in association with hosting a *Blastodinium*.

MATERIALS AND METHODS

NEW OBSERVATIONS ON *BLASTODINIUM* SPP. HOSTS, OCCURRENCE, AND MORPHOLOGY

Unpublished information on new hosts for *Blastodinium* spp. in the Atlantic Ocean (including two undescribed species) originate from the field work described in Skovgaard and Salomonsen (2009). Hitherto unpublished scanning electron microscopy (SEM) observations on *Blastodinium* sp. dinospores were made as part of the study reported by Skovgaard et al. (2007). Most samples for acquisition of new DNA sequences were collected as part of already published studies (Skovgaard and Saiz, 2006; Skovgaard et al., 2007; Skovgaard and Salomonsen, 2009; Alves-de-Souza et al., 2011). Specimens isolates with the prefix "VIL" in **Figure 7** are from Villefranche-sur-mer, France. Following samples were collected the 2 of September 2009 at the site "La marinière": VIL4, VIL57, and VIL59 from *Farranula rostrata*; VIL15, VIL50, and VIL51 from *Corycaeus* sp.; VIL7 from *Triconia* sp.; and VIL8 from *Corycaeus* cf. *ovalis*. VIL49 from *F. rostrata* was collected the 3 of September 2009 at "Le plateau." VIL61 from *Triconia* sp. was collected the 1 of March 2010 at the B site (43°41′10N 7°18′94E). VIL44 from *Corycaeus* sp. was collected the 9 September 2009 at "La marinière." Isolates with the prefix "GA" were collected in the North Atlantic Ocean.

PHYLOGENETIC ANALYSES

Blastodinium spp. were dissected from their host and the DNA extracted as described by Alves-de-Souza et al. (2011). Primers for SSU and PCR conditions are also explained in this reference. Primers used to amplify the ITS region were 5′-GTCGCTCCTACCGATTGAGT-3′ (name ITS-CER-F) in forward and 5′-CAGACAGGCATGTCACCTTC-3′ (name ITS-CER-R) in reverse. PCR conditions were similar to that explained for the SSU. Both SSU and ITS1, 5.8S, and ITS2 amplicons were cloned and sequenced as following the procedure by Alves-de-Souza et al. (2011), and consensus sequences were deduced from the analysis of three different clones. SSU and ITS alignments were

Table 1 | Recognized species of *Blastodinium* and their reported copepod hosts.

Parasite (Author, year)	Hosts	Reference
***Spinulosum* group**	**Calanoida, Cyclopoida, Poecilostomatoida**	
B. spinulosum (Chatton, 1908)	*Acrocalanus gracilis* Giesbrecht	2
	Clausocalanus arcuicornis Dana	1
	C. furcatus Brady	1
	C. farrani Sewell	2
	Paracalanus aculeatus Giesbrecht	2
	P. denudatus Sewell	2
	P. parvus Claus	1
B. pruvoti (Chatton, 1906)	*Clausocalanus arcuicornis*	1
	C. furcatus	1
	Nannocalanus minor Claus	2
	Paracalanus parvus	2
B. crassum (Chatton, 1908)	*Paracalanus parvus*	1
	P. nanus Sars	2^{+}
	Calocalanus styliremis Giesbrecht	1
	Clausocalanus arcuicornis	1
	C. furcatus	1
	P. aculeatus	2^{+}
B. inornatum (Chatton, 1920)	*Clausocalanus arcuicornis*	1
	C. furcatus	1
	Paracalanus parvus	1
B. oviforme (Chatton, 1912)	*Corycaeus crassiusculus* Dana	2
	C. speciosus Dana	2
	Farranula gibbula Giesbrecht	2
	Oithona similis Claus	1
	O. nana Giesbrecht	1
	O. plumifera Baird	1
	Oncaea media Giesbrecht	2
	O. venusta Philippi	2
	Triconia conifera Giesbrecht	2
***Contortum* group**	**Calanoida**	
B. contortum (Chatton, 1908)	*Acartia clausi* Giesbrecht	1
	Acrocalanus gracilis	2
	Calocalanus styliremis	1
	Clausocalanus arcuicornis	1
	C. furcatus	1
	C. lividus Frost & Fleminger	3?
	Cosmocalanus darwini Lubbock	2
	Eucheata indica Wolfenden	2
	Nannocalanus minor	2
	Paracalanus aculeatus	2
	P. denudatus	2
	P. parvus	1
	Subeucalanus pileatus Giesbrecht	2
	S. subtenuis Giesbrecht	5?
	Temora stylifera Dana	**Figures 11E,F**
B. hyalinum (Chatton, 1929)	*Acartia clausi*	4/1
	Calanus finmarchicus Gunnerus	4/1
	Centropages sp.	1
	Clausocalanus arcuicornis	4/1
	C. furcatus	1
	Paracalanus aculeatus	1
	P. denudatus	2
	P. parvus	2
	Paracalanus sp.	1
	Paraeuchaeta antarctica Giesbrecht	4/1
	Pseudocalanus elongatus Boeck	6?
	Pseudocalanus sp.	2
B. apsteini (Sewell, 1951)	*Clausocalanus arcuicornis*	1/2
	C. furcatus	2
	Paracalanus aculeatus	2
B. chattoni (Sewell, 1951)	*Clausocalanus arcuicornis*	2
	C. furcatus	2
	Cosmocalanus darwini	2
	Eucheata indica Wolfenden	2
	Nannocalanus minor	2
	Paracalanus aculeatus	2
	P. denudatus	2
	P. parvus	2
***Mangini* group**	**Poecilostomatoida, Calanoida**	
B. mangini (Chatton, 1908)	*Farranula gibbula* Giesbrecht	$2^{\#}$
	F. rostrata Claus	1*
	Oncaea media Giesbrecht	$2^{\#}$
	O. venusta Philippi	$2^{\#}$
	Oncaea cf. s*cottodicarloi* Heron & Bradford-Grieve	$7^{\#}$
	Triconia conifera Giesbrecht	$2^{\#}$
B. mangini var. *oncaea* (Chatton, 1912)	*F. rostrata*	1
	O. media	1*
	Triconia minuta Giesbrecht	1
B. navicula (Chatton, 1912)	*Corycaeus giesbrechti* Dahl	1
	O. venusta	2*
B. elongatum (Chatton, 1912)	*Centropages* sp.	1
	Scolecithrix bradyi Giesbrecht	1
B. galatheanum Skovgaard	*Acartia negligens* Dana	8
	Acartia sp.	8
***Blastodinium* spp.**		
Blastodinium sp. α	*Temora stylifera*	1
Blastodinium sp. β	*Clausocalanus furcatus*	1
Blastodinium sp. γ	*C. arcuicornis*	1
	Paracalanus parvus	1
Blastodinium sp. δ	*Corycaeus giesbrechti*	1
Blastodinium spp.	*Acrocalanus longicornis* Giesbrecht	5
	Aetidius giesbrechti Cleve	3
	Centropages typicus Krøyer	3
	Clausocalanus furcatus	3
	C. parapergens Frost & Fleminger	10

(Continued)

Table 1 | Continued

Parasite (Author, year)	Hosts	Reference
	Corycaeus flaccus Giesbrecht	3
	C. typicus Krøyer	3
	Euchaeta rimana Bradford	5
	Farranula carinata Giesbrecht	9
	Nannocalanus minor	3
	Neocalanus robustior Giesbrecht	5
	Pareucalanus sewelli Fleminger	5
	Pleuromamma gracilis Claus	3
Blastodinium sp. I	*Euchaeta* sp.	**Figures 6A,B**
Blastodinium sp. II	*Euchaeta* sp.	**Figures 6C,D**

**The genera Oncaea and Triconia have been thoroughly revised since the work of Chatton (1920) and Sewell (1951) signifying that these early observations probably included several at that time unrecognized host species.*

+ No distinction was made between B. crassum and B. inornatum.

 # No distinction was made between B. mangini and B. mangini var. oncaea.

A question mark indicates that the parasite species was identified tentatively by the authors.

1, Summarized by Chatton (1920); 2, Sewell (1951); 1/2, Reported by Chatton (1920) and parasite subsequently identified by Sewell (1951); 3, Ianora et al. (1990); 4/1, Reported by Apstein (1911) and parasites subsequently identified by Chatton (1920); 5, Pasternak et al. (1984); 6, Øresland (1991); 7, Skovgaard (2005); 8, Skovgaard and Salomonsen (2009); 9, Drits and Semenova (1985); 10, Alves-de-Souza et al. (2011).

Species names were validated according to Razouls et al. (2005–2012).

obtained using the online version of MAFFT[1]. ITS alignment was secondarily manually optimized using secondary structures using models described by Gottschling and Plötner (2004). Non-informative sites were removed using Gblocks[2]. A Bayesian phylogenetic tree was constructed with MrBayes v3.2 (Huelsenbeck and Ronquist, 2001) using a GTR substitution model with gamma-distributed rate variation across sites (GTR + I) as suggested as the best-fit model in MrModelstest v2.3 (Nylander, 2004). Four simultaneous Monte Carlo Markov chains were run from random trees for a total of 1,000,000 generations in two parallel runs. A tree was sampled every 100 generations, and a total of 2,500 trees were discarded as 'burn-in' upon checking for stationarity by examination the log-likelihood curves over generations, and posterior probabilities were calculated in MrBayes. A consensus tree (50% majority rule) was constructed from the post-burn-in trees and posterior probabilities were calculated in MrBayes.

TRANSMISSION ELECTRON MICROSCOPY

For transmission electron microscopy (TEM) the intact copepods, *Faranulla rostrata*, with parasites were fixed by two methods: (1) 1.5 ml of 2.5% glutaraldehyde on 0.05 M cacodylate buffer (pH 7.4) diluted from 0.2 M on marine water were mixed with 0.5 ml of 4% OsO_4 and added to the sample for 35 min in the dark. Thus, the final concentrations of glutaraldehyde and OsO_4 were 1.9 and 1% correspondingly. (2) 0.5 ml of 2.5% glutaraldehyde on 0.05 M cacodylate buffer (pH 7.4) diluted from 0.2 M on marine water were mixed with 0.5 ml of 4% OsO_4 and added to the sample for 40 min in the dark (final concentrations of glutaraldehyde and OsO_4 were 1 and 2% correspondingly). The dehydration with alcohol series from 30 to 70% followed without rinsing. All steps of fixation and the dehydration were on ice. The material was kept in 70% alcohol in the freezer (−20°C) for a week before the consecutive dehydration and embedding in Epon. The ultrathin sections were prepared using a ultra-microtome Leica ultracut UCT (Leica Microsystems, Germany), stained with uranyl acetate and lead citrate, and viewed in a JEOL JEM 1400 transmission electron microscope (JEOL Ltd., Japan) at 80 kV, equipped with digital camera Orius SC1000 (Gatan Inc., USA).

PHOTOSYNTHESIS

Photosynthetic rates, P, were measured by the modified single cell technique as described by Skovgaard et al. (2000). Copepods were collected in the NW Mediterranean Sea in November, 2003 (Skovgaard and Saiz, 2006) and used for experiments within the same day. All handling and incubation was done at a temperature corresponding to that of the site of collection (16.2°C). Prior to incubation, copepods (*Oncaea* spp.) were gently picked out individually and rinsed in 0.2-μm filtered, freshly collected seawater. For incubation, copepods were transferred to 20 ml capacity glass scintillation vials containing 2 ml 0.2-μm-filtered seawater. Each vial contained two copepods infected with *Blastodinium* sp. (presumably *B. mangini*). A $NaH^{14}CO_3$ stock solution was added (specific activity = 100 μCi ml^{-1}, Carbon 14 Centralen, DHI-Group, Denmark), resulting in a specific activity of approximately 0.9 μCi ml^{-1}. Vials were then incubated for 4 h in triplicates at irradiances of 50, 150, 250, and 350 μmol photons m^{-2} s^{-1} plus a triplicate that was incubated in the dark. After incubation, specific radioactivity of the medium was checked after incubation by transferring 100 μl incubation water from each vial to new vials containing 200 μl NaOH. The amount of fixed ^{14}C was measured in the remaining 1.9 ml, which received 2.0 ml of 10% glacial acetic acid in methanol to remove all inorganic C. Vials were dried overnight at 60°C whereupon residues were re-dissolved in 2 ml distilled water and 10 ml of Packard Insta-Gel Plus scintillation cocktail (PerkinElmer, USA) were added to all vials. Finally, new caps (Packard poly screw caps, PerkinElmer) were mounted and activities were determined with a Packard 1500 Tri-Carb liquid scintillation analyzer (PerkinElmer). Calculations of photosynthetic rates, P, were based on the equation given by Parsons et al. (1984).

RESULTS AND DISCUSSION

LIFE CYCLE STAGES AND THEIR MORPHOLOGY

Life cycle

The complete life cycle of *Blastodinium* has not been demonstrated definitively, but the morphology of distinct stages of the parasite cycle has been described. According to Chatton (1920), the infection cycle of *Blastodinium* starts with the ingestion of a dinospore by a copepod and, instead of becoming digested, the dinospore grows in size and develops into a trophocyte, which is the earliest parasitic phase that has been identified. In young trophocytes that

[1] http://mafft.cbrc.jp/alignment/server/

[2] http://molevol.cmima.csic.es/castresana/Gblocks_server.html

have recently infected their host, an anterior beak may sometimes be present (**Figure 1B**).

Following infection, the trophocyte produces the characteristic large multicellular structure (**Figure 1**), which corresponds to the parasite undergoing palisporogenetic divisions. Thus, the trophocyte divides into a secondary trophocyte and a gonocyte contained within a common cuticle (**Figure 1D**). This external cuticle of the sporogenetic individual was described by Chatton (1920) to be formed by the delamination of the mother trophocyte membrane (**Figures 2–4**). In comparison with the unitary membrane, this cuticle is much thicker, about 15–20 nm (**Figure 4B**) vs. the thickness of 7–8 nm of the unitary membrane. The cuticle has a three-layered structure: an electron dense inner, a translucent middle, and a comparatively dense outer layer (**Figure 4B**). None of these three layers has a typical membrane structure. Thus, the cuticle can be considered an extracellular envelope. When stained with Calcofluor White (which stains dinoflagellate thecal plates; Fritz and Triemer, 1985), it is the covering of the underlying cells that is stained rather than the cuticle (**Figures 5A,B**). After the initial division of the trophocyte, the produced gonocyte will divide into a large number of sporocytes still retained within the external cuticle, resulting in a large multicellular individual (Chatton, 1920; **Figures 1** and **5A,B**). In some cases (to some extent species dependent) the secondary trophocyte will divide into a tertiary trophocyte, and a new gonocyte will then produce a second layer of sporocytes (**Figure 1F**). This process may be repeated and result in several layers of sporocytes. Chatton (1920) referred to these conditions as mono-, di-, or polyblastic, dependent on how many layers of sporocytes were surrounding the trophocyte. In some species, the trophocyte is not completely embedded by sporocytes, leaving a "naked" area, a hilum (**Figures 1E** and **2A**), where the trophocyte is visible and in direct contact with the cuticle.

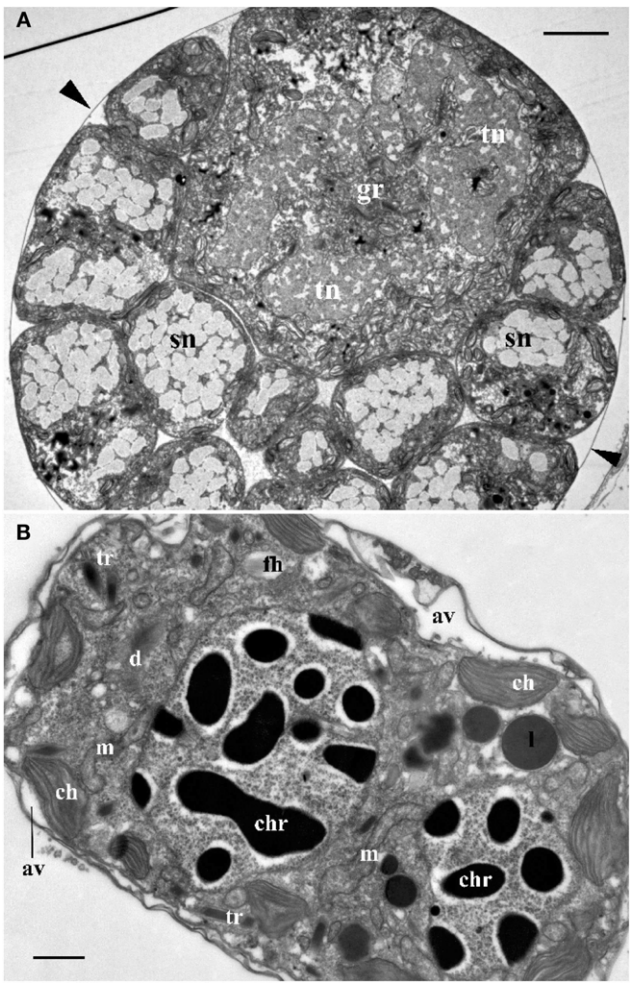

FIGURE 2 | (A) Cross section through the central part of *Blastodinium* cf. *mangini* (from *Farranula rostrata*) showing a big trophocyte with several layers of sporocytes covered by common a cuticle (arrowheads). gr, golgi region; sn, nucleus of sporocyte; tn, nucleus of trophocyte. Scale bar = 5 μm. **(B)** Ultrastructure of mature *Blastodinium* cf. *navicula* sporocyte (from *F. rostrata*). av, amphiesmal vesicle (alveolus); ch, chloroplast; chr, chromosome; cu, cuticle; d, dictyosome; fh, vesicle with future flagellar hairs; l, lipid droplet; m, mitochondrion; tr, trichocyst. Scale bar = 1 μm.

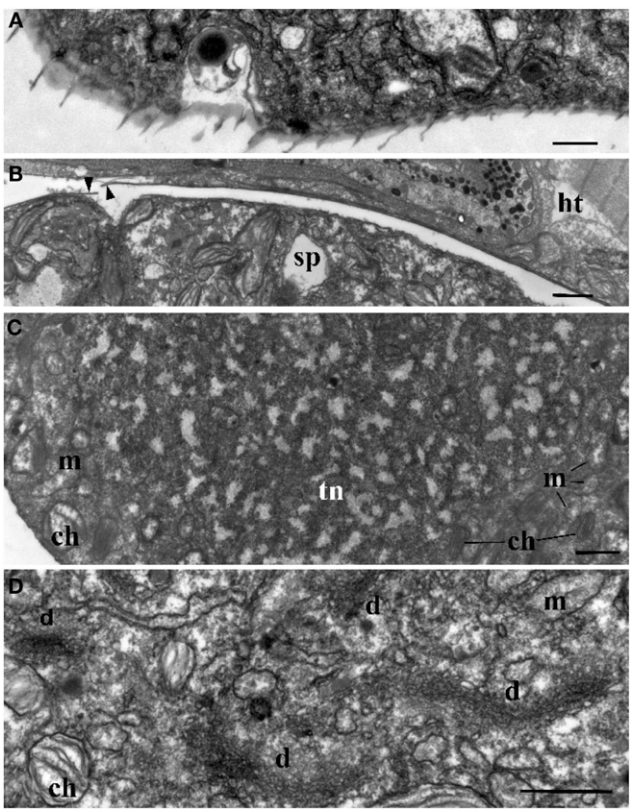

FIGURE 3 | Ultrastructure of *Blastodinium*. (A) Cell covering of *Blastodinium* cf. *mangini* (from *Farranula rostrata*) in crest region. **(B)** Cuticle of trophont attached tightly to the gut tissue of the host (ht); arrowheads show broken cuticle on the left. *Blastodinium* cf. *mangini* (from *F. rostrata*). **(C)** Portion of trophocyte of *Blastodinium* cf. *navicula* sporocyte (from *F. rostrata*) with huge nucleus (tn) containing decondensed chromosomes (light zones) and granular material (possibly ribosomal subunits), small chloroplasts (ch) and mitochondria (m). **(D)** Golgi region of trophocyte of *B.* cf. *mangini* (from *F. rostrata*) with prominent dictyosomes (d), chloroplasts (ch), and mitochondria (m). Scale bar = 1 μm.

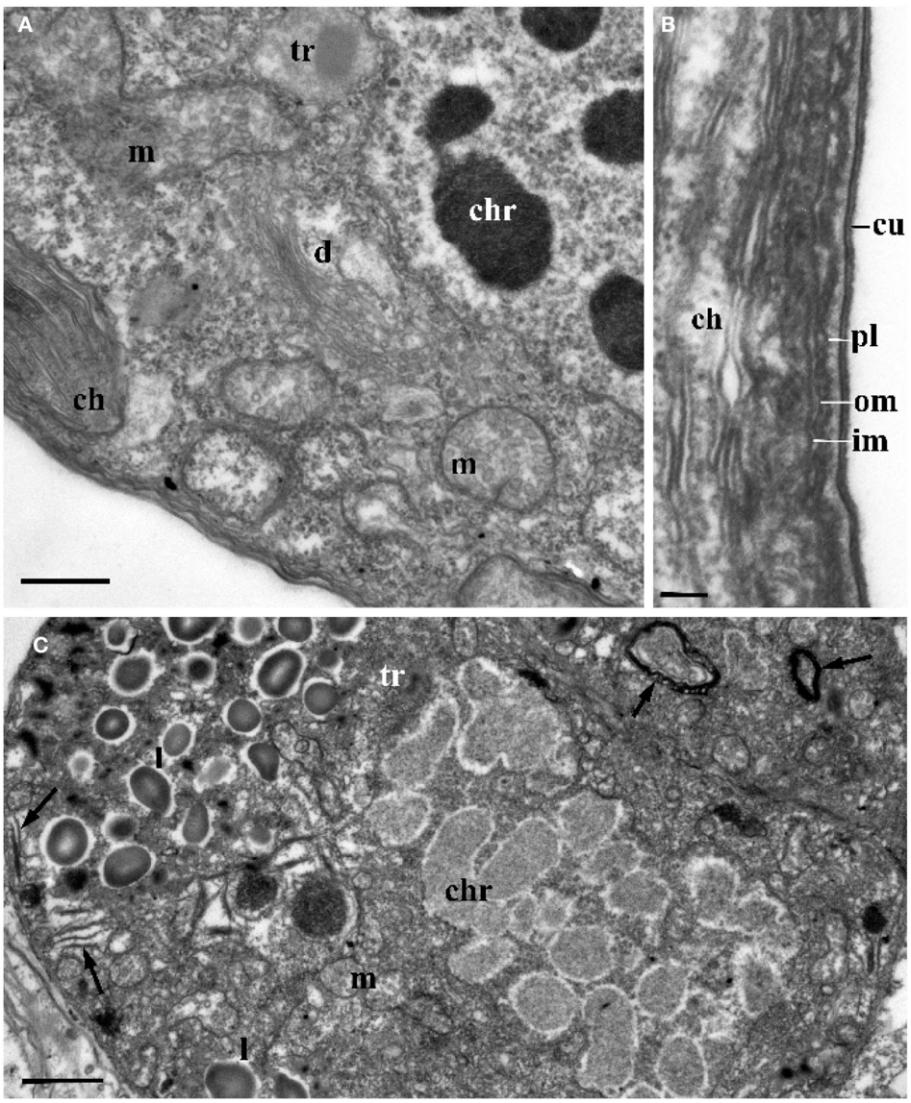

FIGURE 4 | The ultrastructure of *Blastodinium* sporocyte. (A) Portion of nucleus and cytoplasm of *Blastodinium* cf. *navicula* sporocyte (from *F. rostrata*). **(B)** Structure of coverings of *Blastodinium* cf. *navicula* sporocyte (from *F. rostrata*). **(C)** Colorless representative of *Blastodinium* (*Blastodinium* cf. *hyalinum* from *Paracalanus parvus*). ch, chloroplast; chr, chromosome; cu, cuticle; d, dictyosome; im, inner and outer (om) membrane of alveoli; l, lipid droplets; m, mitochondrion; pl, plasma membrane; tr, maturing trichocyst. Arrows show reduced presumed plastids. **(A)** Scale bar = 2.5 μm. **(B)** Scale bar = 0.1 μm. **(C)** Scale bar = 1 μm.

Sporulating individuals generally measure up to several hundreds of μm in length and are often detected coincidentally inside the transparent copepod's gut thanks to their size and the brownish to greenish color caused by the presence of chloroplasts. The rupture of the cuticle leads to the release of sporocytes into the water through the copepod anus.

The surface of the parasite cuticle is at times ornamented with two helical rows of minute spinules along the body (**Figure 1C**). These "helicoidal crests" can be difficult to discern in the light microscopy, but are readily seen in SEM (Skovgaard and Salomonsen, 2009) and in ultrathin sections (**Figure 3A**). They are formed by papillae located on the trophocyte (see Figure XXVII, p. 132, in Chatton, 1920). The crests may also be visualized by hematoxylin-staining (Coats et al., 2008). The crests are not one continuous helix, but formed by two distinct sections. This reflects the fact that the trophozoite is composed of "Siamese twins," each of them having their own nucleus (Chatton, 1920). This dualism is also conserved in sporocytes (**Figure 2B**). *Blastodinium* spp. do not possess a peduncle or other holdfast organelles (Fensome et al., 1993), but one may speculate that the anterior beak of young trophocytes and/or the spinules of the helicoidal crests may play a role in anchoring the parasite to the copepod gut lining. In addition, sections made on infected copepods show that sporulating *Blastodinium* appear attached to the gut epithelium, and the outer covering has a tight contact with epithelium (**Figure 3B**).

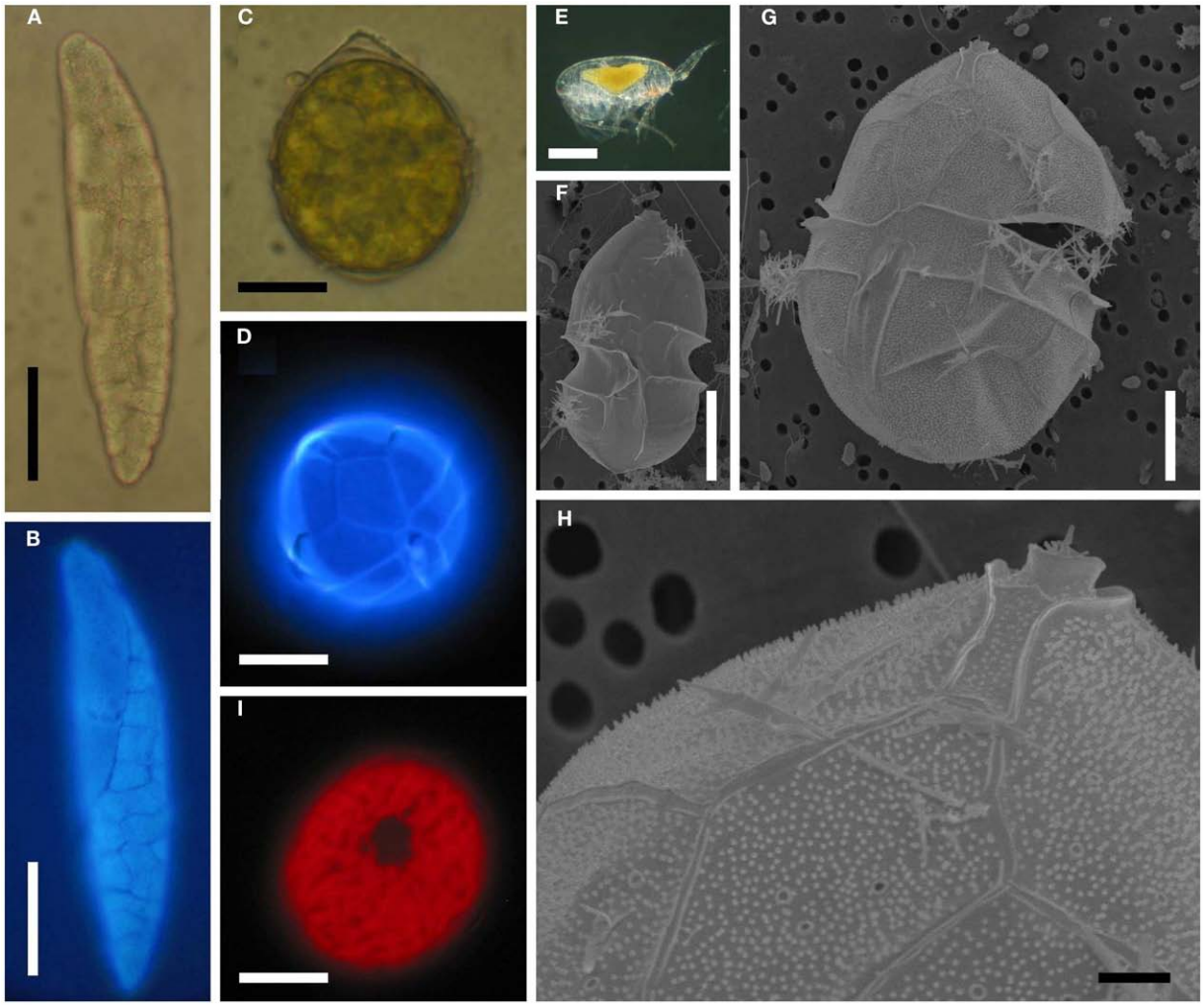

FIGURE 5 | *Blastodinium* spp. (A) *B. mangini* from *Oncaea* sp. **(B)** Same as **(A)** but stained with Calcofluor White. **(C)** *B. oviforme* dinospore from *O. similis*. **(D)** Same as **(C)** but stained with Calcuflour White. **(E)** *Clausocalanus* sp. infected with *Blastodinium* sp. **(F)** *B. contortum* dinospore, SEM. **(F)** *B. contortum* dinospore, SEM. **(G,H)** Dinospore from the parasite in **(E)**, SEM. **(I)** *Blastodinium* sp. dinospore showing autofluorescence of chloroplasts viewed in epifluorescent light. **(A,B)** Scale bar = 50 μm. **(C,D,I)** Scale bars = 10 μm. **(E)** Scale bar = 200 μm. **(F,G)** Scale bars = 5 μm. **(H)** Scale bar = 1 μm.

Some *Blastodinium* species are gregarious, i.e., several individuals of the same parasite are present in one host individual. Such gregarious parasites are thought be the result of a division of the trophocyte by binary fission into two daughter trophocytes (either before or during sporogenesis). Chatton (1920) used the term "endogenous multiplication" for this type of division compared to the sporogenesis that was referred as "exogenous multiplication" or "palisporogenesis." During endogenous multiplication the two daughter trophocytes are each surrounding by a new cuticle, and they will eventually produce independent, but synchronous, generations of sporocytes. Rupture of the original cuticle will then release two daughter parasites of approximately similar size. Conversely, the infection with one *Blastodinium* species appears to prevent succeeding infections (Chatton, 1920). Multiple individuals in a single host are, thus, typically of approximately similar developmental stage. However, this is not always the case, and Sewell (1951) consequently suggested that successive infections of a single host may also be possible. The final proof of multiple infections would be the presence of two different *Blastodinium* species in a single host, but this has never been observed.

The dinospore

Sporocytes released through the copepods anus are initially non-motile. After few hours, these bi-nucleated cells acquire flagella and divide into four uni-nucleate dinospores (Chatton, 1920; Soyer, 1971; **Figure 5C**). These dinospores are thecate with plates that are clearly visible when stained with Calcofluor White (**Figure 5D**) as well as in SEM (**Figures 5F–H**). The pattern and arrangement of thecal plates on *Blastodinium* dinospores is typical for Peridiniales. A pattern of pores encircled by each a single ring gives some resemblance to the peridinoid dinoflagellate genus *Pentapharsodinium* (Skovgaard et al., 2007) on which thecal plates are ornamented with one or two concentric rings. With respect of the thecal tabulation pattern, no distinct differences have been found between

the morphology of the species *B. navicula*, *B. contortum*, and *B. galatheanum* (Skovgaard et al., 2007; Skovgaard and Salomonsen, 2009). However, variation in the morphology of *Blastodinium* dinospores does occur. The cell depicted in **Figure 5G** is considerably larger than the typical dinospore (**Figure 5F**). Even though the plate tabulation pattern is similar in both types of dinospores, the larger dinospore has a distinctive theca possessing a dense coverage of papillae (**Figures 5G,H**). These unusual dinospores were collected after having been expelled from *Blastodinium* sp. hosted by a single specimen of *Clausocalanus* sp. (**Figure 5E**) and they were motile at the time of fixation. Thus the aberrant morphology of these dinospores cannot presently be explained, but it is probable that they represent a species yet not described.

Infection and transmission

While it appears logical that *Blastodinium* infects its host through oral transmission by a dinospore, the means of infection has never been proved experimentally. Likewise, the mechanism by which the infective dinospore is subsequently able to avoid digestion and remain in the gut of its host is currently unknown. Attempts to transmit *Blastodinium* experimentally from infected copepods to uninfected individuals have been unsuccessful (Chatton, 1920; Skovgaard, 2005). It has been suggested that adult copepods are not infected, but that infection takes place in the juvenile stages (Chatton, 1920; Alves-de-Souza et al., 2011). This theory might explain the failure of transmission experiments, since these have concentrated on late copepodite stages and adult copepods.

ULTRASTRUCTURAL MODIFICATIONS DURING SPOROGENESIS

The *Blastodinium* trophocyte has an aberrant morphology compared to typical dinoflagellates, which is plausibly a result of its parasitic life style. Following successive sporogenetic generations, cells are gradually re-acquiring typical features of free-living dinoflagellates. These morphological transformations can be observed in a single polyblastic individual, since such individual will have different layers of sporocytes of different age (Soyer, 1970, 1971).

Nucleus

Ultrastructure of the nucleus during mitotic divisions was studied in detail by Soyer (1971). According to that report and **Figures 2–4**, *Blastodinium* has a dinokaryotic nucleus at all stages with the nuclear envelope remaining intact during mitosis and chromosomes staying attached to the inner membrane. Invaginations of the nuclear envelope with cytoplasmic microtubules inside are also frequently observed, demonstrating typical dinomitosis. However, although the trophocyte nucleus has dinokaryotic chromosomes (lacking histones), these are decondensed with a large amount of granular contents (ribosomal subunits) around (**Figures 2A** and **3C**). Progressive condensation of chromosomes takes place during sporogenesis (Soyer, 1971). The first sporocyte layers have nuclei with less nucleoplasm and much more condensed chromosomes. They will remain in such condition during several sporogenetic divisions (**Figures 2A** and **4A,C**). The most condensed chromosomes appear in the mature sporocytes (**Figure 2B**) and this chromosome compaction is concomitant to the reduction of nuclear size.

Chloroplasts

Plastids of the trophocyte are rather small, often with light stroma and few thylacoids (like etioplastids in plants; Soyer, 1970; **Figures 2A** and **3C,D**). However, they are fully reactivated during the course of sporogenesis and in mature sporocytes the plastids are well developed and located at the cell periphery (**Figure 2B**). Pyrenoids are present in later stage only (**Figures 2B** and **4A**). The colorless species *B. hyalinum* seems to possess remnants of chloroplasts, but these are very rare and appear to be highly degenerated (Soyer, 1970; **Figure 4C**).

Trichocysts

Blastodinium has typical dinoflagellate trichocysts that are very rare, if present at all, in the trophocyte (**Figure 3**). Some premature stages of trichocyst development are found in immature sporocytes (**Figure 4**), and many well-developed trichocysts are present in mature, binucleate sporocytes (**Figure 2B**).

Golgi apparatus

Soyer (1970) also reported additional transformations in the Golgi apparatus and mitochondria along the sporogenetic process. These observations are not confirmed here in sections of *B. mangini*, but **Figures 2** and **3D** show that the Golgi apparatus was extremely large in the trophocyte, composed of several huge dictyosomes, some of them up to 5 μm in length. It occupies a big region between the two nuclei of the trophocyte (Chatton, 1920; **Figures 2** and **3D**). Both the nuclear structure and the Golgi structure reveal the intense metabolic activity of the trophocyte stage. In addition, mitochondria were well developed at all stages of *Blastodinium* proliferation having typical dinoflagellate tubular cristae (**Figures 3** and **4**).

Cell covering

Membrane structures surrounding the trophocyte and sporocytes are of similar appearance, being covered by three membranes corresponding to the typical dinoflagellate amphiesma (**Figure 4**). The alveoli are flat with electron translucent contents. The outer membranes of the alveoli attach tightly to the plasma membrane. However, this amphiesma becomes more elaborate in mature sporocytes, with broader alveoli (**Figure 2B**). In conclusion, a mature sporocyte with two nuclei has very condensed chromosomes, developed amphiesma, mature trichocysts, and prominent chloroplasts (**Figure 2B**), which seem to represent typical features of naked immature dinospores.

TAXONOMY

Taxonomic position of the genus

Since the discovery of *Blastodinium*, it has been recognized that these organisms exhibit features that separate them from the bulk of the dinoflagellates of the class Dinophyceae. *Blastodinium* has thus been appointed as the type genus of a separate class, the Blastodiniphyceae (Fensome et al., 1993), comprising the single order Blastodiniales. Blastodiniphyceae was synonymized with Haplozooidea and placed in the superclass Hemidinia by Cavalier-Smith (1993). The main character distinguishing the class from the Dinophyceae has been a parasitic life mode and the presence of histones in larger trophic cells and the absence of such in the smaller swarmers (dinospores), i.e., a temporary dinokaryon (Fensome et al.,

1993). Recent findings that *Blastodinium* dinospores are thecate with a thecal plate tabulation pattern corresponding to that of Peridinian dinoflagellates (Skovgaard et al., 2007) suggest a closer relationship with the Dinophyceae and this is also supported by molecular phylogeny.

Species distinction

The main characters for species discrimination within the genus *Blastodinium* are based upon morphological distinctions of the parasitic stage (Chatton, 1920), such as size, the location of the trophocyte, coloration, number of sporocyte layers, the presence of a hilum, and the existence of helicoidal crests. Another important character is whether the parasites are solitary or gregarious in their hosts. Among the gregarious species, also the number of parasites in each host is given taxonomic importance. Gregarious species typically have 2, 3, or 4 parasites in each host, but some species, such as *B. spinulosum*, are often more numerous; more than 10 is not unusual and up to 23 parasites have been found in a single *B. spinulosum* in *Paracalanus parvus* (Chatton, 1920). Based on these morphological characters, Chatton (1920) arranged *Blastodinium* spp. in three groups, the *spinulosum* group, the *contortum* group (in which the two species described by Sewell also fit), and the *mangini* group (**Table 1**). The main characteristics of the *spinulosum* group are that the parasites are curved and shaped like a small boat with a rounded anterior pole and a pointed posterior pole, the trophocyte is located in the anterior part, and the parasite body is traversed by a groove and one or more helicoidal crest(s). The *contortum* group is characterized by the parasite body being twisted in early developmental stages, they have no helicoidal crest, no groove, and they are usually solitary in their host. In the *mangini* group, both poles of the parasite are rounded and individuals are nearly straight. Finally, the host species, or the range of host species, is of taxonomic importance.

There are currently 13 accepted species of *Blastodinium* (**Table 1**), of which the majority were discovered in copepods from the Mediterranean Sea early in the twentieth century (Chatton, 1906, 1908, 1911, 1912, 1920). Two taxa were originally described as varieties (*B. crassum* var. *inornatum* and *B. contortum* var. *hyalinum*; Chatton, 1920) but these have subsequently been generally accepted as independent species, i.e., *B. crassum* and *B. hyalinum*. A couple of species were afterward found in the Arabian Sea and described by Sewell (1951). Since then only a single new species has been identified and described, namely *B. galatheanum* (Skovgaard and Salomonsen, 2009). *B. hyalinum* is the only species that was explicitly described as being colorless (Chatton, 1911). However, the two species named by Sewell (1951), *B. apsteini* and *B. chattoni*, were noted as having closest similarity to *B. hyalinum*, so even though the pigmentation was not mentioned in the description of these two species, one must assume that they were considered to be colorless.

Unrecognized morphological diversity

Limited work has been done on the taxonomy of *Blastodinium* since the work by Chatton and Sewell, and studies of *Blastodinium* outside European waters are still few. It is, therefore, possible that the diversity within this genus is not yet fully elucidated, and indeed several morphotypes have been observed which cannot be assigned to any known species (**Table 1**). A study on *Blastodinium* in the Mediterranean Sea revealed several specimens with a morphology that did not match any described species (Alves-de-Souza et al., 2011). The isolate BOUM7 in that study was not only morphologically different from any known *Blastodinium* species; it was also genetically distinct from other members of the genus. Furthermore, during a recent 2-weeks cruise in warm, oligotrophic waters of the central Atlantic Ocean from the Azores Islands to the Southern coast of West Africa, a new *Blastodinium* species was found and described (Skovgaard and Salomonsen, 2009) and a couple of specimens of each two other unknown *Blastodinium* specimen were observed (**Figure 6**). One of these was a large species (>1 mm long) with six conspicuously colored parasites in a single host individual, *Euchaeta* sp. (**Figures 6A,B**); the other one a solitary likewise colored specimen in the same host species (**Figures 6C,D**). The ease by which these unknown members of *Blastodinium* were found reinforces perceptions that the morphological diversity of the genus is presently being underestimated. In fact, scientists tend not to assign species names to individual organisms when studying *Blastodinium* spp. (Pasternak et al., 1984; Ianora et al., 1990; Øresland, 1991), which probably reflects the high morphological variation within the genus resulting in many morphotypes that appear intermediate between recognized species.

PHYLOGENY

Phylogenetic position of the genus

The temporary dinokaryon has been considered the phylogenetically important character distinguishing the Blastodiniphyceae from the typical dinoflagellates, the Dinophyceae (Saunders et al., 1997), and the Blastodiniphyceae have been considered evolutionary basal to the Dinophyceae. The importance of the temporary dinokaryon as a taxonomic feature has, however, been questioned (Fensome et al., 1993) and its phylogenetic value may also be limited. Furthermore, molecular phylogenetic studies have shown that species originally classified in the Blastodiniphyceae are polyphyletic and have placed several blastodinian dinoflagellate species well within the Dinophyceae (Saldarriaga et al., 2004). Hence, differences in nuclear structure are of dubious phylogenetic significance.

Traditional dinoflagellate morphology-based taxonomy does not always conform to modern taxonomy in which molecular phylogeny is taken into account (Fensome et al., 1999; Saldarriaga et al., 2004) and the phylogeny of dinoflagellates has been emended considerably after molecular phylogeny has been widely incorporated (e.g., Daugbjerg et al., 2000). This also applies to the blastodinian dinoflagellates. While *Blastodinium* has shown affinity to the Peridiniales in molecular phylogenic analyses (Skovgaard et al., 2007), other blastodinian species branch elsewhere within the Dinophyceae (Saldarriaga et al., 2004). According to molecular phylogenetics, Blastodiniphyceae, and Blastodiniales thus have no validity, a fact that may not be surprising when considering the peridinian morphology of the dinospores. However, a formal revision of the taxonomic position of *Blastodinium* has yet to be made and such revision may need to await a more general revision of the Peridiniales and related dinoflagellates.

Even though emerging SSU rDNA sequence data confirms a close taxonomic relation between individual *Blastodinium* species,

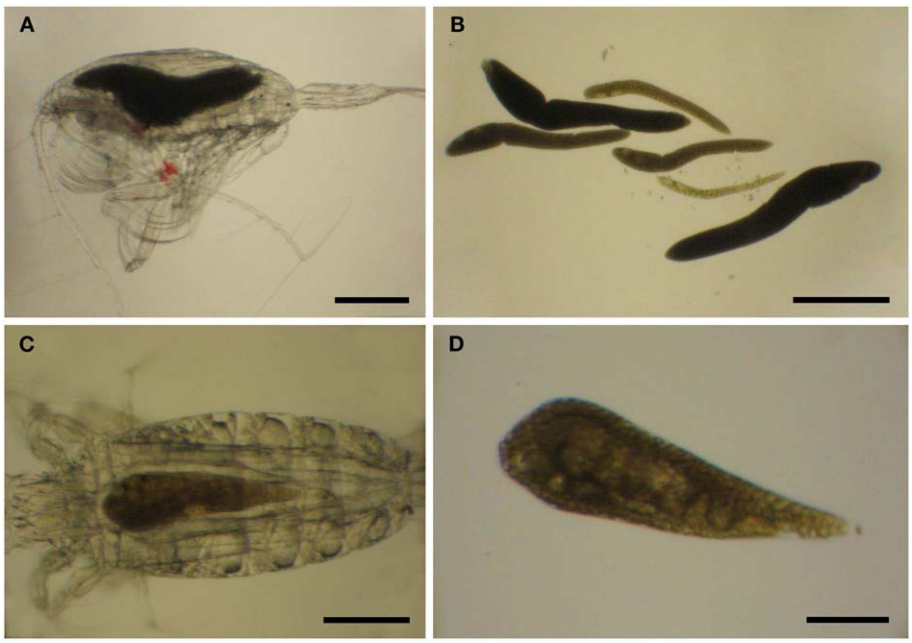

FIGURE 6 | *Blastodinium* sp. in *Euchaeta* sp. from the Atlantic Ocean. (A,C) Parasites inside their hosts. **(B)** Six parasites of an unidentifiable species from the host in **(A)**. **(C)** A single unidentifiable parasite from the host in **(C)**. **(A,B)** Scale bars = 500 μm. **(C)** Scale bar = 200 μm. **(D)** Scale bar = 100 μm.

these are not always monophyletic in phylogenetic analysis (Skovgaard et al., 2007; Alves-de-Souza et al., 2011). A couple of recent analyses have, on the other hand, shown *Blastodinium* to be monophyletic, but the support for this is consistently low (Coats et al., 2008; Skovgaard and Salomonsen, 2009). All these reports have, nevertheless, agreed that *Blastodinium* belong phylogenetically to the typical, dinophycean dinoflagellates.

Intrageneric phylogeny

Phylogenetic analyses (Maximum Likelihood, ML, and Bayesian inference, BI) of *Blastodinium* spp. based on 18S rDNA and ITS (ITS1, ITS2, and 5.8S rDNA) sequences are presented in **Figure 7**. Some of the more characteristic specimens sequenced for these analyses are depicted in **Figures 8** and **9**, including the two newly sequenced species *B. inornatum* and *B. oviforme*. These analyses do not address the potential of lack of monophyly among *Blastodinium* spp., since only *Blastodinium* sequences (including putative *Blastodinium* sequences) are included. Overall, the two data sets (18S rDNA vs. ITS) show consensus with moderate to high support for the *contortum*, *spinulosum*, and *mangini* groups (**Figure 7**), indicating that gross morphology does reflect molecular phylogeny within the genus. This is despite the fact that the two data sets are based in part on different samples. The *mangini* group has the poorest resolution among the three major groups, and has high support only in the ITS analyses (ML bootstrap value of 97 and BI posterior probability of 1.00). In the *mangini* group, only *B. oviforme* and *B. navicula* ITS sequences branch out as monophyletic. On the contrary, the positions of *B. mangini* (both trees) and *B. galatheanum* (ITS tree) do not agree exactly with the morphology-based classification, and in the 18S phylogeny *B. navicula* is not well resolved. In particular *B. mangini* sequences are very diverse and this harmonizes with the high morphological variation described by Chatton (1920), leading him to erect *B. mangini* var. *oncaea*. The isolate BOUM7 (*Blastodinium* sp.) clusters together with the *mangini* group with a long branch. Indeed, in a previous analysis including also a number of non-*Blastodinium* dinoflagellates, the BOUM7 isolate branched out separately from the other *Blastodinium* clades (Alves-de-Souza et al., 2011).

The consistent high support for the *contortum* group suggests that these sequences may in fact comprise a single species (**Figure 7**), which is here represented from different host species and different locations (Mediterranean Sea and Gulf of California). The *spinulosum* group, on the contrary, is as a whole well supported, but it is very diverse and the individual clades do not conform entirely with the identification of species as determined through morphological traits. Most typical *B. spinulosum* morphotype sequences do branch in one clade (Group *spinulosum* I), but this clade also encompasses *B. pruvoti*, *B. inornatum*, and *B. crassum* morphotypes (**Figure 7**). On the other hand, *B. spinulosum* morphotype sequences are also present in the *spinulosum* II clade. One of these *B. spinulosum* morphotypes (BCL01) was morphologically distinct by comprising eight individuals of which two were apparently colorless (**Figure 9G**).

In conclusion, it is probable that both the *mangini* group and the *spinulosum* group(s) encompass one or more species complexes and that, possibly, a larger species diversity than currently recognized exists within *Blastodinium*. A high genetic diversity within *B. mangini* was to be expected considering the large number of hosts known for this species and the fact that the morphology of *B. mangini* can be quite variable (Chatton, 1920). Unfortunately, sequences from *B. hyalinum*, *B. chattoni*, and *B. apsteini*

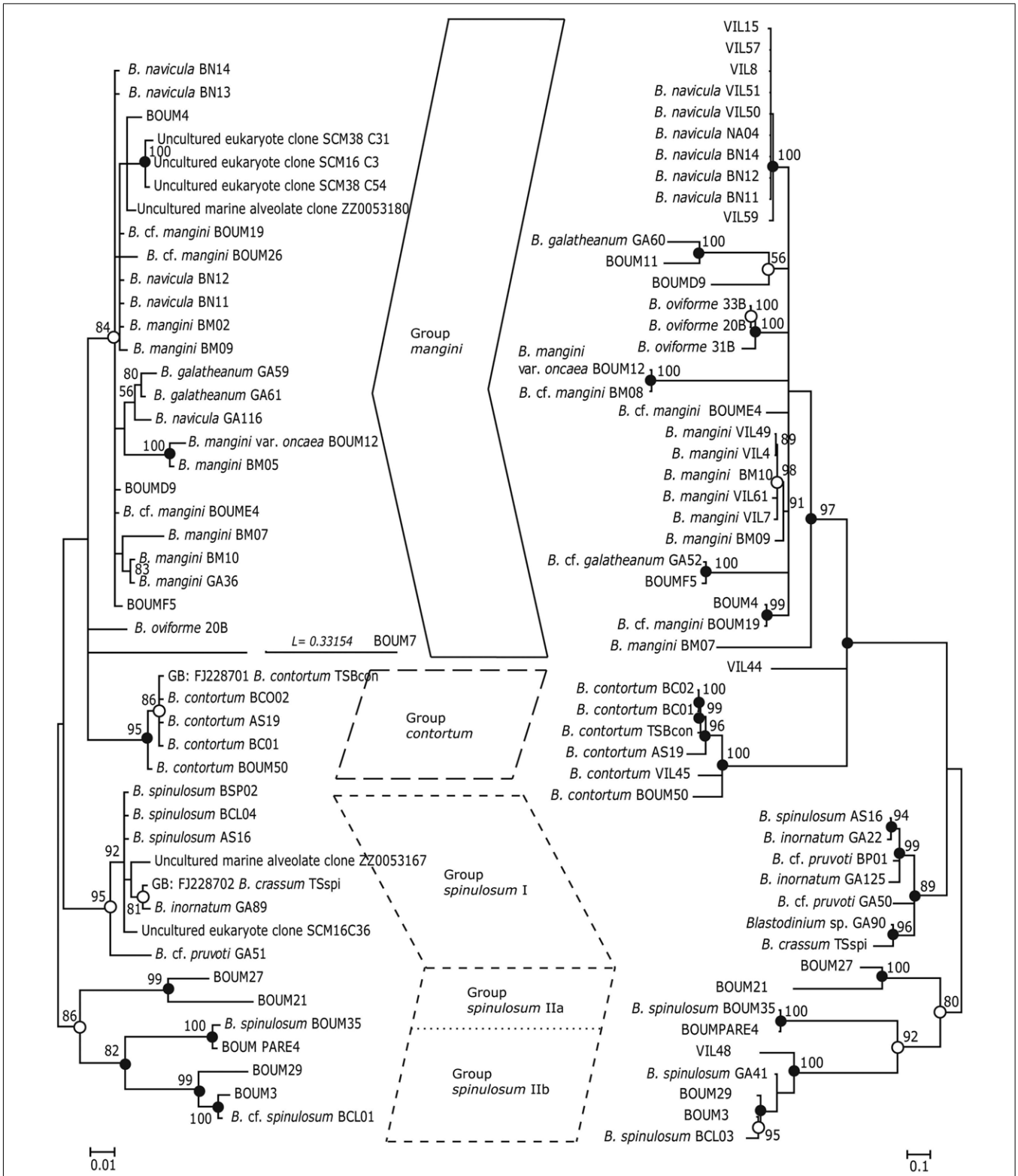

FIGURE 7 | Phylogenetic trees based on Bayesian analyses of alignments comprising SSU rDNA sequences (left) and ITS1, 5.8S rDNA, and ITS2 sequences (right) of *Blastodinium* spp. Numbers above nodes are Maximum Likelihood bootstrap values; only values above 50% are shown. Filled circles at nodes denote that the clade had Bayesian posterior probabilities (PP) of 1.00; open circles denote PP of 0.95–0.99. PP < 95 are not shown. Labels at branches are species names and/or isolate names. Two sequences are identified by their GenBank accession number; these have the prefix "GB:" GenBank accession numbers for all sequences are given in **Table A1** in Appendix.

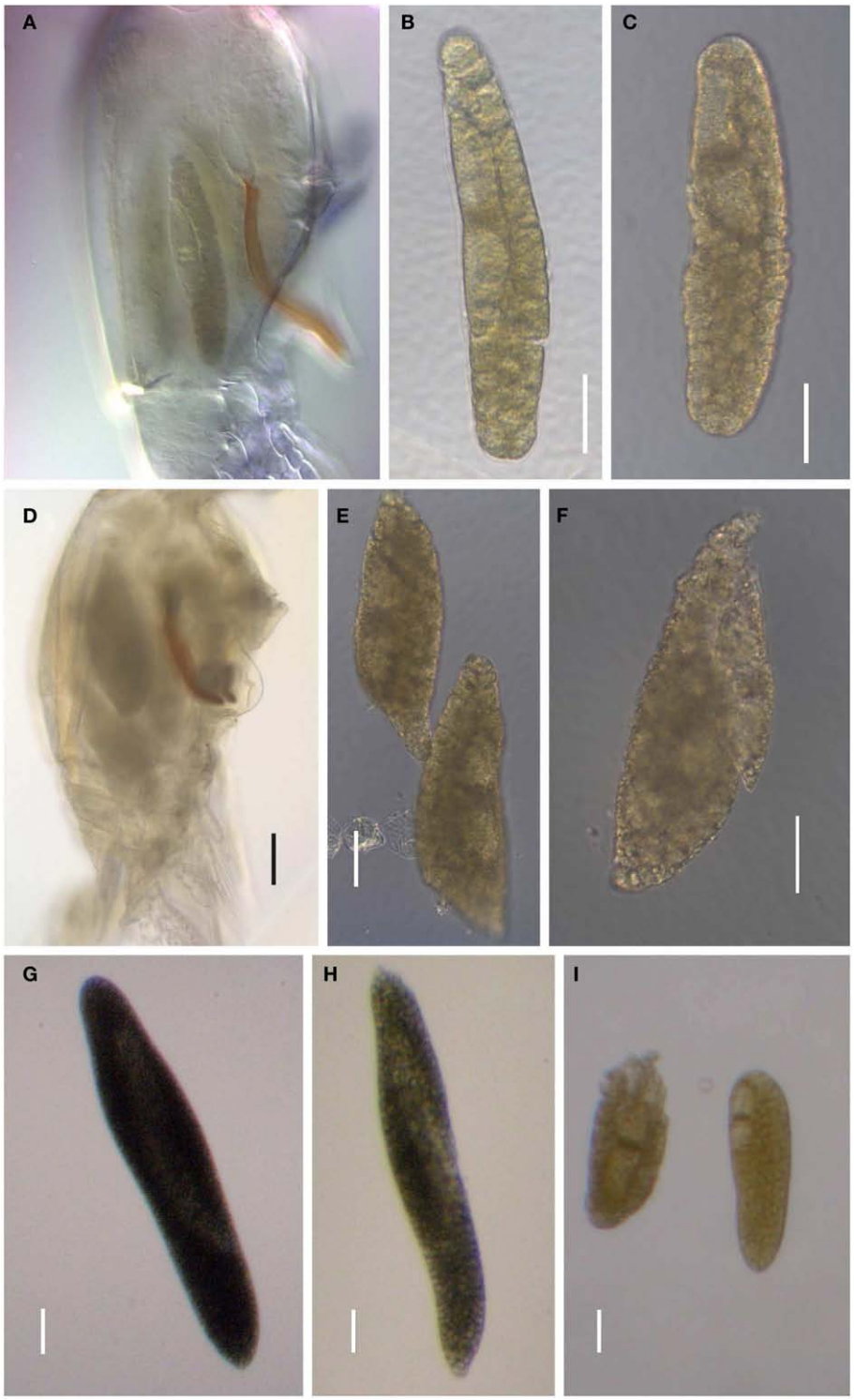

FIGURE 8 | *Blastodinium* spp. (A–C) *Blastodinium mangini*. **(A)** Three individuals inside *Farranula rostrata* (Villefranche-sur-mer, 2 of September 2009). Monoblastic stage (I-128). **(B)** Individual extracted from *F. rostrata* (Villefranche-sur-mer, 3 of September 2009, VIL49). Monoblastic stage (I-64). **(C)** Individual extracted from *F. rostrata* (Villefranche-sur-mer, 2 of September 2009, VIL52). Monoblastic stage (I-128). **(D–F)** *B. navicula*. **(D)** Four individuals inside *F. rostrata* (Villefranche-sur-mer, 2 of September 2009, VIL50). **(E,F)** Different individuals extracted from the precedent copepod host (VIL50). Monoblastic stage (I-128). **(G)** *Blastodinium* cf. *galatheanum* from *Acartia negligens* (North Atlantic, GA52). **(H)** *B. galatheanum*. from *A. negligens* (North Atlantic, GA60). Scale bar = 50 μm. I. *B. mangini* var. *oncaea*. Two individuals from *Oncaea* sp. (NW Mediterranean Sea, BM05). **(B,C)** Scale bars = 50μm. **(D)** Scale bar = 100 μm. **(E–I)** Scale bars = 50μm. **(G)**

FIGURE 9 | *Blastodinium* spp. Sequences from samples are used for the phylogenetic analyses in **Figure 7**. **(A–C)** Solitary individual of *B. inornatum* Group *spinulosum* I from *Clausocalanus* spp. **(A)** GA22. **(B)** GA89. **(C)** GA125. **(D–F)** Gregarious individuals of *Blastodinium* spp. Group *spinulosum* I from *Clausocalanus* spp. **(D)** *Blastodinium* cf. *pruvoti*, BP01. **(E)** *Blastodinium* cf. *pruvoti*, GA50. **(F)** *B. oviforme* from *Oithona similis*. **(G,H)** Gregarious individuals of *B. spinulosum* Group *spinulosum* II. **(G)** *Blastodinium* cf. *spinulosum*, BCL01, from *Clausocalanus* cf. *arcuicornis*. Note two colorless individuals. **(H)** *B. spinulosum*, GA41, from *Paracalanus parvus*. **(I)** *B. spinulosum*, BCL04, Group *spinulosum* I from *Clausocalanus* sp. Scale bars = 100 μm.

are currently not available and the phylogenetic position of these colorless species is, thereby, unknown.

OCCURRENCE

Species distribution

Reports on *Blastodinium* spp. suggest that the genus occurs worldwide in seawater with close to full strength salinity, i.e., more than approximately 30 ppt. (**Figure 10**). Most observations on *Blastodinium* have been done in coastal waters, but this pattern is likely to reflect the ease of access to sampling sites rather than the actual distribution of the organisms. Based on the work by Apstein (1911) and subsequent observations, Chatton (1929) noted that *B. hyalinum* was the only species present in the cold temperate North Sea, whereas a row of green (photosynthetic) species existed in warm temperate waters of the Mediterranean Sea. This observation concurs with that of subsequent studies,

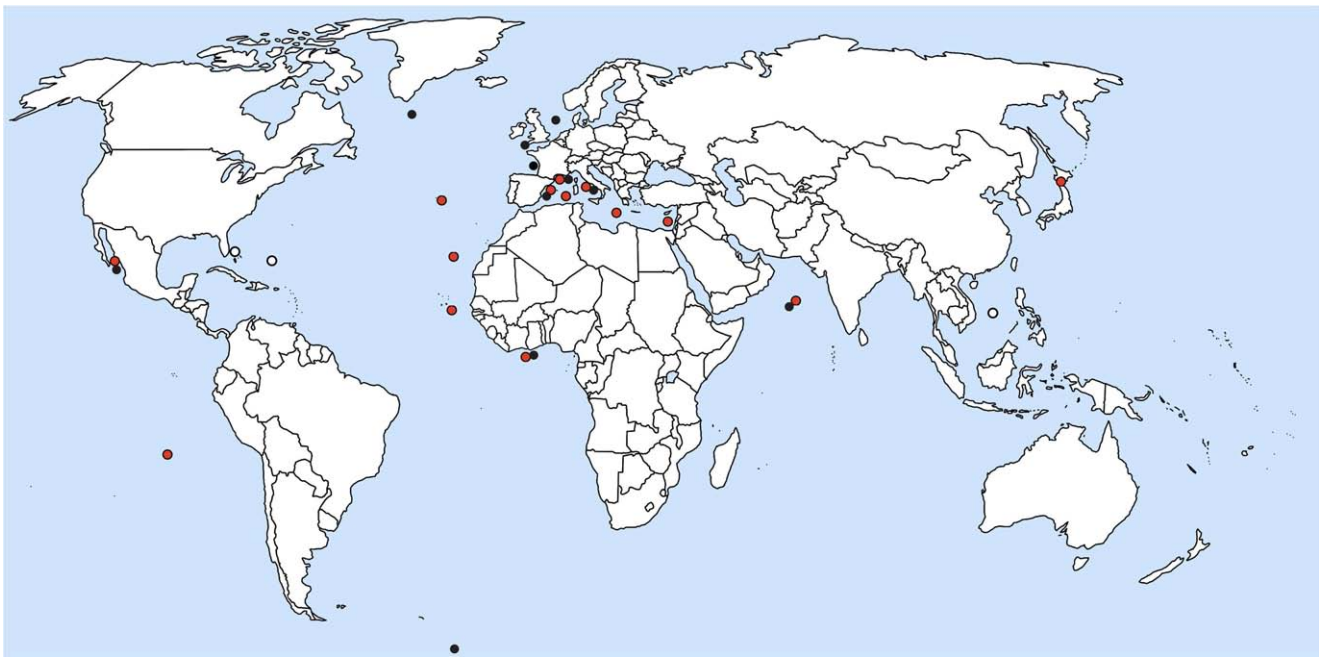

FIGURE 10 | Location of current reports of *Blastodinium* spp. in marine copepods. Red circles represent photosynthetic species; black circles are colorless species; and white circles are environmental DNA sequences with high similarity to *Blastodinium*.

finding exclusively *B. hyalinum* in cold temperate waters (Lebour, 1925; Jepps, 1937; Vane, 1952). Also the species reported by Øresland (1991) in *Euchaeta antarctica* from Antarctic waters was presumably *B. hyalinum* considering the length of the parasite (2.5–3.5 mm; Øresland, 1991), matching no other known *Blastodinium* species. As further support for *B. hyalinum* being a world-wide species, **Figures 11A,B** depict *B. hyalinum* in *Calanus* sp. from Greenlandic waters. On the other hand, photosynthetic species of *Blastodinium* are restricted to warm temperate, subtropical, and tropical waters. These waters are often oligotrophic suggesting that the life strategy of *Blastodinium* spp. has adapted to such an environment. One may speculate that a semi-parasitic organism, a "parasitic alga," as *Blastodinium* will benefit from being able to acquire inorganic nutrient from its host in oligotrophic waters, thereby avoiding potential nutrient limitation. Considering the relatively small number of surveys, it is to be expected that both the geographic range and the host range of *Blastodinium* are broader than now recognized. A further addition to the known geographic range of *Blastodinium* cf. *chattoni* is given in **Figures 11C,D**, showing this parasite in *Cosmocalanus vulgaris* collected in the Central Atlantic Ocean off the West coast of Africa by Skovgaard and Salomonsen (2009). From the same waters, a rare example of a *Temora stylifera* was also found infected with a *Blastodinium* (**Figures 11E,F**), in this case a species that was identified as an early developmental stage of *B. contortum*.

Occurrence of dinospores

Free-swimming *Blastodinium* cells (dinospores) are rarely encountered in the marine plankton. This may be because dinospores are short-lived and therefore less likely to be detected, but it is also possible that *Blastodinium* dinospores are overlooked due to their superficial resemblance with free-living, non-parasitic peridinoid dinoflagellates. Alves-de-Souza et al. (2011) found numerous dinospores in the Mediterranean Sea using DNA-probes and proposed that some of the "small thecate dinoflagellates" often observed in mixed plankton samples may in fact be *Blastodinium* dinospores. Also, SSU rDNA sequences from Sargasso Sea nanoplankton show high similarity with *Blastodinium* spp. and are most likely to originate from *Blastodinium* dinospores (Skovgaard and Salomonsen, 2009). The same may apply for two ITS1 sequences from the South China Sea (GenBank accession numbers GU941876 and GU942050). Little is known about the vertical distribution of *Blastodinium* spp. dinospores, but Alves-de-Souza et al. (2011) found maximum densities of dinospores at or just below the deep chlorophyll maximum in the Mediterranean Sea (approximately 100 m), i.e., in an environment where photosynthetic growth is unlikely to be of any significance. Occurrence of *Blastodinium* spp. is seasonal (Chatton, 1920; Skovgaard and Saiz, 2006) suggesting that the life cycle may contain a dormant stage. Indeed, Chatton (1920) mentioned the presence of cysts, but their fate and function remain unknown.

Host-specificity

Most *Blastodinium* species infest several copepod host species. This was originally realized on the basis of *Blastodinium* morphology (Chatton, 1920; Sewell, 1951) and it is corroborated by recent ITS sequences. For example sequences of isolate VIL61 from *Triconia* sp. were identical to VIL4 and VIL49 (**Figure 8B**) from *F. rostrata.* However, there is a clear distinction between species that infect calanoid copepods and those that infect cyclopoid copepods. This pattern was noted already by Chatton (1920, p. 236) and has been corroborated in subsequent investigations (**Table 1**). Also, there

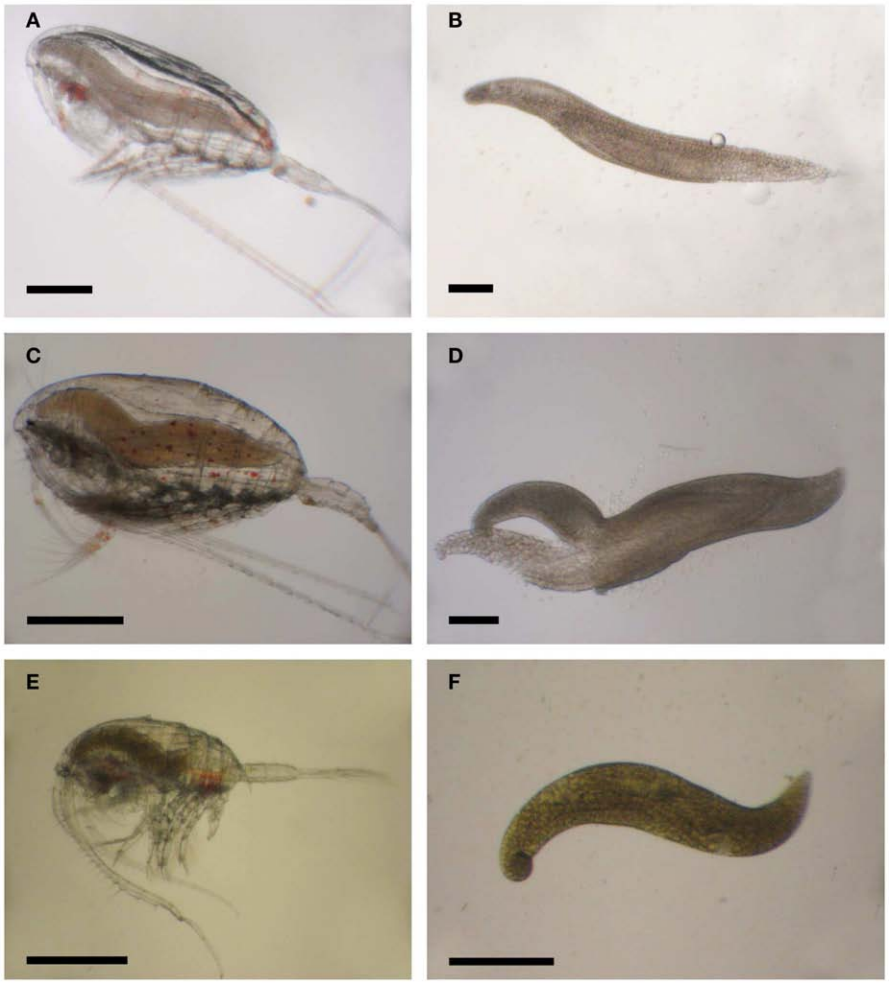

FIGURE 11 | *Blastodinium* spp. in calanoid copepods. (A) *Calanus* sp. from Greenlandic waters with *B. hyalinum*. **(B)** Parasite from **(A)**. **(C)** *Cosmocalanus vulgaris* off the West coast of Africa with *Blastodinium* cf. *chattoni*. **(D)** Parasite from **(C)**. **(E)** *Temora stylifera* from the Central Atlantic Ocean with *Blastodinium* cf. *contortum*. **(F)** Parasite from **(E)**. **(A,C,E)** Scale bars = 500 μm; **(B,D,F)** scale bars = 200 μm.

is a large variation in the number of host species a single *Blastodinium* species is able to infect, even though this observation may depend on how commonly the parasite species is observed. It is interesting to note that several common copepod species, such as *Oithona nana* and *T. stylifera*, are rarely infected with *Blastodinium* spp. in mixed plankton communities in which several other copepod species have high infection prevalence (Skovgaard and Saiz, 2006).

ECOLOGY

Photosynthetic growth

The gut of planktonic copepods has been shown to be a suitable environment for photosynthesis (Epp and Lewis, 1981) and since most *Blastodinium* species possess well-developed chloroplasts it is natural to assume that they are capable of photosynthesis. Pasternak et al. (1984) demonstrated that this is in fact the case and made the crude estimate that *Blastodinium* sp. was able to fulfill approximately half of its energy needs through photosynthesis, implying that the other half must somehow be obtained from the host. To further test this, the photosynthetic rate of *Blastodinium* cf. *mangini* hosted by *Oncaea* spp. (**Figure 12A**) was determined in November in the NW Mediterranean Sea (**Table 2**). The photosynthetic rate, P, was up to 826 pg C parasite^{-1} h^{-1} corresponding to a P per volume of the parasite of 1.6 ng C cm^{-3} h^{-1}. At the lower irradiance of 50–150 μE m^{-2} s^{-1}, P/vol was 0.6–0.9 ng C cm^{-3} h^{-1}. The magnitude of P may be put in perspective by comparing with P of mixotrophic free-living dinoflagellates with a known relative contribution of photosynthesis for cell growth: P/vol of *Gyrodinium resplendens* was 2.5 ng C cm^{-3} h^{-1} at 75 μE m^{-2} s^{-1} (Skovgaard, 2000), and P/vol of *Fragilidium subglobosum* was 2.8 ng C cm^{-3} h^{-1} at 150 μE m^{-2} s^{-1} (Skovgaard et al., 2000). Hence, the photosynthetic activity of *Blastodinium* cf. *mangini* is less than half of that of the two free-living, mixotrophic dinoflagellates. *F. subglobosum* acquired only 10% of its C needs through photosynthesis under the conditions at which P was measured (Skovgaard et al., 2000), and *G. resplendens* acquired approximately 16% of its C demand through photosynthesis. Assuming that all factors are equal, *Blastodinium* cf. *mangini* should then

Table 2 | Photosynthetic rates, P, of *Blastodinium* sp. inside *Oncaea* sp. at four different irradiances.

Irradiance (μmol photons m^{-2} s^{-1})	P[a] (pg C parasite^{-1} h^{-1})	SE ($n = 3$)	P/vol[b] (ng C μm^{-3} h^{-1})
50	400	–	0.6
150	509	6	0.9
250	574	26	1.1
350	826	13	1.6

[a] *Each replicate contained two copepods hosting each two parasites, i.e., measured P was four times that reported here.*

[b] *P per volume of parasite. Volume calculated according to a prolate ellipsoid. Average dimension of parasites used: L = 200 μm, W = 70 μm.*

acquire an even smaller fraction of its C needs through photosynthesis. According to this crude approximation, the estimate that *Blastodinium* sp. should fulfill approximately half of its energy needs through photosynthesis (Pasternak et al., 1984) is not unrealistic, but may be a comparatively high estimate. The existence of *Blastodinium* species with apparently non-functional chloroplasts (i.e., the colorless species) gives good reason to believe that *Blastodinium* spp. are able to obtain a substantial part of their energy needs heterotrophically through organic substances acquired from the host.

Blastodinium dinospores also contains chloroplasts (**Figure 5I**), suggesting a potential for photosynthetic growth. This could hypothetically be an advantage for the dispersal of the parasite, since dinospores would be able to stay alive for longer time outside their host. However, in a culture experiment, *B. mangini* dinospores did not survive for longer than a week, regardless whether they were incubated in light or in the dark (Skovgaard, 2005). Yet, in the optimum environment one would still expect a beneficial effect of chloroplasts for the survival time of dinospores and this could potentially increase the probability of finding a new suitable host. It is also possible, on the other hand, that the main photosynthetic activity occurs in the sporocytes, thereby supporting growth of the parasite inside the host. According to this hypothesis, the chloroplasts in dinospores may be a vestige from the preceding photosynthetic stage.

EFFECTS ON THE HOST

Growth and fitness

Infection with *Blastodinium* spp. generally leads to a reduced size of the infected host (Chatton, 1920; Sewell, 1951; Alves-de-Souza et al., 2011). This may be caused by food limitation induced by the parasite physically blocking the alimentary tract, but it is also possible that the parasite is utilizing organic matter in the gut, thereby competing with the host for the ingested food. Infected copepods also have a decreased survival as compared to uninfected copepods, which is possibly caused by the same food limitation mechanisms (Skovgaard, 2005). One could speculate that hosting a photosynthetic organism would be advantageous for the host and that the parasite would a beneficial symbiont supplying its host with organic matter. However, a comparison of survival of infected vs. uninfected *Oncaea* spp. incubated both in light and in the dark did not reveal any difference and thus did not give any support for *Blastodinium* being beneficial to its host (Skovgaard, 2005).

Castration

Blastodinium spp. is able to induce castration of infected female copepods (Chatton, 1920; Sewell, 1951; Skovgaard, 2005) and gonads are usually not fully developed in infected organisms (Chatton, 1920). Incubation experiments have demonstrated that infected copepods usually do not deposit eggs (Ianora et al., 1990; Skovgaard, 2005) and, even though infected females have been found carrying eggs at a few occasions (Vane, 1952; Skovgaard, 2005), hatching of eggs produced by an infected female copepod has never been witnessed. On the other hand, histological and ultrastructural examinations of *Blastodinium*-infected copepods, have shown ovaries and reproductive diverticulae to be normal in size and shape and oogonal development appeared comparable to uninfected individuals (Ianora et al., 1990). It thus appears that an infection with *Blastodinium* spp. does not necessarily destruct the gonad anatomy, as is the case with the more detrimental parasite *Syndinium turbo* (Ianora et al., 1990). The infection does, however, lead to physiological castration, which is probably induced by the parasite "stealing" energy from its host and/or by the mechanical pressure exerted by the parasite on the reproductive organs. Some parasitic castrators are able to modify the scheme by which the host allocates energy, thereby shunting resources from reproduction to growth (Hall et al., 2007). This mechanism is not likely to be of importance for *Blastodinium* spp. given the fact that infected copepods are smaller than healthy specimens.

Female vs. male infection

In general, only adult females and juveniles are infected with *Blastodinium* spp. Chatton (1920) found a large number of female and male copepodites to be infected, but did not find a single infected, adult male. He ascribed this to a failure of infected male copepodites of molting to the adult stage. Later studies have shown *Oncaea* spp. to be an exception to be above rule, since male *Oncaea* spp. are frequently infected with *B. mangini* (Sewell, 1951; Skovgaard, 2005) and so are males of species within Corycaeidae (Alves-de-Souza et al., 2011). Infections in adult males of other species are extremely rare: Sewell (1951) found a single adult *Nanocalanus minor* male to be infected with *B. contortum*. This represents the only of two examples of *Blastodinium* infection in an adult calanoid male, the other example being a male *Acartia clausi* observed by Ianora et al. (1990).

The prevailing hypothesis predicts that *Blastodinium* spp. are transmitted through oral infection. Such entry of infection would explain the lack of infection in adult males, since males of many copepod species are short-lived and do not have the capability to feed, in particular among the Clausocalanoidea (Ohtsuka and Huys, 2001). If infected, juvenile males are unable to molt to the adult stage; this would efficiently prevent the existence of infected males. *Oncaea* spp. are, on the other hand, among those species in which males do feed (Ohtsuka et al., 1996). Hence, the pattern of infection in males supports the hypothesis of oral transmission of infection. There are no records of behavioral changes in copepods due to infection with *Blastodinium*

spp. On the contrary, males do mate with infected females (Cattley, 1948) and, in the case of *Oncaea* spp., both infected males and females have been observed mating, and even two infected individuals will mate with each other (Skovgaard, 2005; **Figure 12B**).

Sex alternation

Copepods parasitized by *Blastodinium* spp. have been observed to be morphologically intersexual (Jepps, 1937; Cattley, 1948; Sewell, 1951; Ianora et al., 1987) and this has been interpreted as parasite-induced sex reversal (Cattley, 1948). However, the proof that parasitism is a main causal factor for intersexuality and sex reversal in copepods is non-existent, since sex alternation is common among copepods – also among non-parasitized individuals (Shields, 1994). Furthermore, intersex is caused by several factors other than parasitism, e.g., mortality, differential longevity, and environmental factors such food as limitation (Gusmão and McKinnon, 2009). Nevertheless, it does appear that intersexuality is more common among copepods parasitized by *Blastodinium* spp. than among non-parasitized copepods (Sewell, 1951 and parasitism should, therefore, be considered among the environmental factors causing intersexes in copepods (Gusmão and McKinnon, 2009).

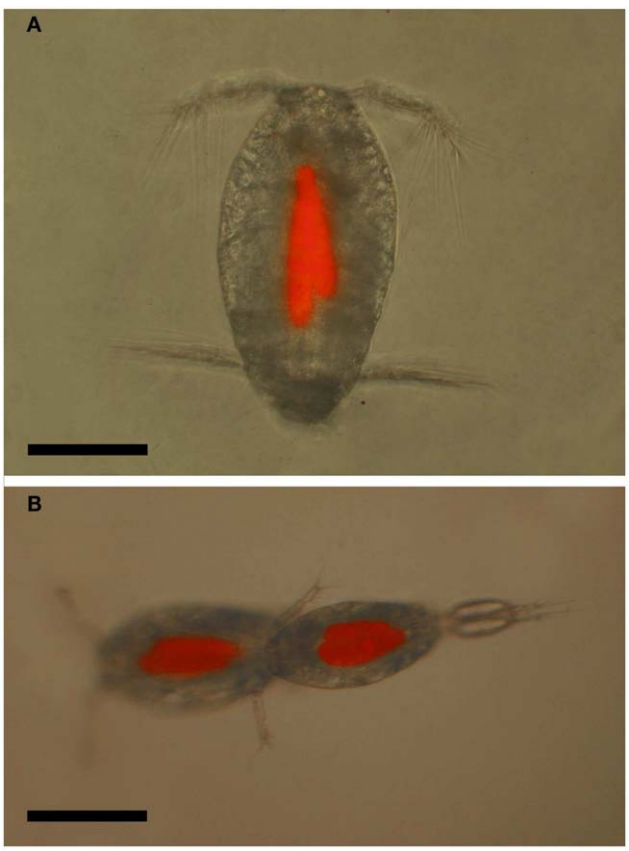

FIGURE 12 | ***Oncaea* cf. *scottodicarloi* with *Blastodinium mangini* in epifluorescent illumination showing autofluorescence of parasite chloroplasts. (A)** Female with two parasites. **(B)** Copulation male (right) and female (left), both infected with 2–3 parasites. Scale bars = 100 μm.

Prevalence

Most current knowledge on the occurrence, diversity, and prevalence of *Blastodinium* spp. is from the Mediterranean Sea and this is also the only site from where seasonal data are available. These data show marked seasonal variations and demonstrate that prevalence and diversity of the genus are highest from mid-summer through autumn; *Blastodinium* spp. are rare in late winter and during spring (Chatton, 1920; Skovgaard and Saiz, 2006). Quantitative investigations on *Blastodinium* spp. from the Mediterranean Sea and the Arabian Sea indicate that it is not unusual to find peaks among adult females of some copepod species with 20% or more infected (**Table 3**), but a prevalence below 5–10% is more common (Chatton, 1920; Sewell, 1951; Skovgaard and Saiz, 2006) and some copepod species are infected only to a very low extent. Limited quantitative data is available from cold temperate waters. Vane (1952) found overall infection rates of 3.7 and 3.8% for adult and stage V females of *Calanus finmarchicus* and *Pseudocalanus elongatus*, respectively, from the Continuous Plankton Recorder samples taken from the North Sea (Hardy, 1941). Maximum infection rates were noted to be as high as 66 and 60%, respectively, but sample sizes were, unfortunately, not specified. Other studies have found low numbers of parasites in the North Sea and adjacent waters (Apstein, 1911; Jepps, 1937).

Given the diversity and host-specificity of *Blastodinium* spp., a considerable effort is needed to assess the existence of all *Blastodinium* species in their respective potential hosts, and as a consequence, prevalence is often known only from sporadic investigations and often mainly for copepod species that tend to have highest degree of infection. This could imply that the general prevalence is overestimated. However, the opposite is more likely to be the case, because early developmental stages of parasites are almost certainly systematically overlooked and, furthermore, parasites are typically looked for in preserved samples in which the parasite have lost its color and become less conspicuous. The detection of endoparasites can be facilitated by fixation and storage of samples under conditions that preserve the color of the parasite (Skovgaard and Saiz, 2006), but even under optimum conditions early parasite stages are in risk of being overlooked. A solution to this problem may be to apply cell or DNA stains. Unspecific DNA dyes allow for a rapid detection of the DNA-rich nuclei of *Blastodinium* cells. A recent study used DNA-staining to detect infections and found relatively high infection prevalences; 51 and 33% for Corycaeidae and Calanoidae, respectively (Alves-de-Souza et al., 2011). It may also be possible to stain *Blastodinium* spp. differentially by use other standard staining techniques, such as hematoxylin. The practical feasibility of such staining techniques for quantitative samples is, however, in need for future validation.

Effect on host populations

Little is known about the impact of *Blastodinium* parasitism on natural copepod populations. The direct adverse effect of *Blastodinium* on fitness and survival of the infected host has been documented (Skovgaard, 2005), but has not been accounted for in field investigations. A single study has explored the adverse effect of *Blastodinium* spp. on the population of two copepod species. In this case it was estimated that impairment of the reproductive rate of *Oncaea* cf. *scottodicarloi* females infected with *B. mangini*

Table 3 | Prevalence of *Blastodinium* spp. in different copepod hosts.

Parasite	Host	Prevalence(%)	Location	Reference
Blastodinium crassum	*Paracalanus parvus*	1.5	W Mediterranean Sea	1
B. contortum	*Subeucalanus subtenuis**	6–12	SE Pacific Ocean	2
	P. parvus	0–3.9	W Mediterranean Sea	6
B. hyalinum	*Calanus finmarchicus*	3.7–66	North Sea	3
	Pseudocalanus elongatus	3.8–60	North Sea	3
	P. elongatus	0.3–20.5	North Sea	4
	Paraeuchaeta antarctica^{+}	6.6	Weddel Sea	5
B. mangini	*Oncaea* cf. *scottodicarloi*	0–17	W Mediterranean Sea	6
	Farranula rostrata	10	W Mediterranean Sea	1
B. navicula	*Corycaeus giesbrechti*	20–30	W Mediterranean Sea	1
B. oviforme	*Oithona* spp.	0–3	W Mediterranean Sea	6
B. spinulosum	*P. parvus*	0.4–0.7	W Mediterranean Sea	1
	Clausocalanus spp.	0.9	W Mediterranean Sea	1
Blastodium spp.	*P. parvus*	0.4	W Mediterranean Sea	7
	Calanoida	33$^{\#}$	E Mediterranean Sea	8
	Corycaeidae	51$^{\#}$	E Mediterranean Sea	8
	Oithonidae	<2$^{\#}$	E Mediterranean Sea	8
	Oncaeidae	<2$^{\#}$	E Mediterranean Sea	8

**, The parasite was presumably B. contortum; $^{+}$, the parasite was presumable B. hyalinum; $^{\#}$, based on DNA-stain screening.*

1, Chatton (1920); 2, Pasternak et al. (1984); 3, Vane (1952); 4, Cattley (1948); 5, Øresland (1991); 6, Skovgaard and Saiz (2006); 7, Ianora et al. (1987); 8, Alves-de-Souza et al. (2011).

was 0.05–0.16 day^{-1} and for *P. parvus* females infected with *B. contortum* the impairment was up to 0.03 day^{-1} (Skovgaard and Saiz, 2006). The magnitude of reproductive impairment was concluded to be comparable in effect on host populations to that of sources of predator-induced mortality. *Oncaea* spp. males are also infected to a considerable extent (Sewell, 1951; Skovgaard, 2005, Figure 10B) and infected male and female copepods appear to copulate actively (**Figure 12B**), but possibly copulating with an infected male is never successful, thereby reducing a healthy female's probability of reproducing successfully with a fertile male. Thus, in addition to the parasite-induced castration of females, copulations in which any of the mates are infected will reduce the overall mating success rate, and infection of males will lead to an enforced reduction in this mating success. This mechanism may have a considerable effect on the recruitment success in individual copepod populations.

CONCLUDING REMARKS

Based on the above compilation of historical and recent data combined with new data presented here, it can be concluded that the genus *Blastodinium* is both morphologically and genetically very diverse. It is, thus, certain that the current number of described species does not reflect the actual diversity of the genus: several *Blastodinium* morphotypes and gene sequences cannot be assigned to any known species. Furthermore, DNA sequences from some of these aberrant morphotypes are highly divergent (such as the BOUM7 isolate; Alves-de-Souza et al., 2011). Therefore, the genus *Blastodinium* is not always monophyletic in phylogenetic analyses encompassing the entire Dinokaryota (Alves-de-Souza et al., 2011) and in those analyses that show a monophyletic origin of the genus, the support is typically negligible (Coats et al., 2008; Skovgaard and Salomonsen, 2009). Finally, as shown in the present study (**Figures 7–9**) there is only partial agreement between the morphological distinction of *Blastodinium* species and their separation in molecular phylogenetic analyses. This suggests that cryptic speciation exists within the genus and that morphological characters may be insufficient for the separation of individual species.

Even though most *Blastodinium* species contain functional plastids, there is no evidence of any beneficial effects for a copepod hosting a *Blastodinium*. As shown here, chloroplasts are not fully developed in the trophocyte and, thereby, seem to primarily play a role in the growth of sporocytes. However, as discussed above, *Blastodinium* individuals are conceivably highly dependent on organic matter acquired from the host and this energy flow may be a major cause of the harmful effects recorded on infected copepods. The effects of a *Blastodinium* infection is not detrimental to individual copepod hosts, but infection does lead to decreased fitness and physiological castration, which have the potential to significantly influence the affected copepod populations.

An accumulating number of investigations of the occurrence of *Blastodinium* spp. in the World's oceans strengthen earlier presumptions that photosynthetic species are common but restricted to warm oligotrophic oceans. On the other hand, the colorless species, at least *B. hyalinum*, appear to occur world-wide. Yet, most zooplankton studies do not report on the presence of *Blastodinium* because parasites are generally not considered in standard zooplankton counts and they are easily overlooked by the untrained eye. Nevertheless, the prevalence and effects of *Blastodinium* infections justify that these organisms should ideally be taken into account when assessing zooplankton productivity processes and an important task in future research will be to develop techniques that facilitate registration of parasites in routine zooplankton investigations.

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APPENDIX

Table A1 | GenBank accession numbers for the sequences used in Figure 7.

Species	Isolate	Host	Number of *Blastodinium* ind. per host	Sampling site	SSU	ITS
B. contortum						
B. contortum	AS19	*Paracalanus parvus*	1	NW Med. Sea	DQ317536	JX473668
B. contortum	BC01	*Clausocalanus arcuicornis*	1	NW Med. Sea	DQ317537	JX473669
B. contortum	TSBcon	*Paracalanus parvus* cf.	1	Gulf of California	FJ228701	FJ228701
B. contortum	BOUM50	Calanoida	1	Med. Sea	JN257680	JX473687
B. contortum	BCO02	*Nanocalanus minor*	1	NW Med. Sea	JX473667	JX473667
B. contortum	VIL45	*Paracalanus parvus*	1	Med. Sea	—	JX473688
B. crassum						
B. crassum	TSspi	*Paracalanus parvus* cf.	1	Gulf of California	FJ228702	FJ228702
B. galatheanum						
B. galatheanum	GA59	*Acartia negligens*	1	Atlantic Ocean	FJ541187	—
B. galatheanum	GA61	*A. negligens*	1	Atlantic Ocean	FJ541188	—
B. galatheanum	GA60	*Acartia* cf. *danae*	1	Atlantic Ocean	—	JX473670
B. cf. *galatheanum*	GA52	*A. negligens*	1	Atlantic Ocean	—	JX473671
B. inornatum						
B. inornatum	GA89	*Clausocalanus* sp.	1	Atlantic Ocean	HQ226069	—
B. inornatum	GA22	*Clausocalanus* sp.	1	Atlantic Ocean	—	JX473672
B. inornatum	GA125	Calanoida	1	Atlantic Ocean	—	JX473673
B. mangini						
B. mangini	BM02	*Oncaea* sp.	3	NW Med. Sea	JX473655	—
B. mangini	BM05	*Oncaea* sp.	2	NW Med. Sea	JX473656	—
B. mangini	BM07	*Oncaea* sp.	2	NW Med. Sea	JX473657	JX473674
B. cf. *mangini*	BM08	*Oncaea* sp.	2	NW Med. Sea	—	JX473675
B. mangini	BM09	*Oncaea* sp.	nd	NW Med. Sea	JX473664	JX473664
B. mangini	BM10	*Oncaea* sp.	nd	NW Med. Sea	JX473658	JX473676
B. mangini	GA36	*Oncaea* sp.	3	Atlantic Ocean	JX473659	—
B. mangini	VIL49	*Farranula rostrata*	3	Med. Sea	—	JX473689
B. mangini	VIL4	*Farranula rostrata*	3	Med. Sea	—	JX473690
B. mangini	VIL61	*Triconia* sp.	2	Med. Sea	—	JX473691
B. mangini	VIL7	*Triconia* sp.	2	Med. Sea	—	JX473692
B. cf. *mangini*	BOUM19	*Farranula* cf. *rostrata*	3	Med. Sea	JN257674	JX473701
B. cf. *mangini*	BOUME4	*Farranula* cf. *rostrata*	nd	Med. Sea	JN257677	JX473702
B. cf. *mangini*	BOUM26	Not determined	nd	Med. Sea	JN257676	—
B. navicula						
B. navicula	BN11	*Corycaeus giesbrechti*	nd	NW Med. Sea	DQ317538	JX473677
B. navicula	BN12	*C. giesbrechti*	nd	NW Med. Sea	JX473665	JX473665
B. navicula	BN13	*C. giesbrechti*	nd	NW Med. Sea	JX473660	—
B. navicula	BN14	*C. giesbrechti*	nd	NW Med. Sea	JX473661	JX473678
B. navicula	GA116	*Corycaeus furcifer*	8	Atlantic Ocean	JX473662	—
B. navicula	NA04	*C. giesbrechti*	4	NW Med. Sea	—	JX473679
B. navicula	VIL50	*Corycaeus* sp.	4	Med. Sea	—	JX473693
B. navicula	VIL51	*Corycaeus* sp.	7	Med. Sea	—	JX473694
Blastodinium sp.	VIL15	*Corycaeus* sp.	1	Med. Sea	—	JX473695
Blastodinium sp.	VIL57	*Farranula rostrata*	5	Med. Sea	—	JX473696
Blastodinium sp.	VIL8	*C.* cf. *giesbrechti*	5	Med. Sea	—	JX473697
Blastodinium sp.	VIL59	*Farranula rostrata*	4	Med. Sea	—	JX473698
B. oviforme						
B. oviforme	20B	*Oithona* sp.	1	NW Med. Sea	JX473666	JX473666
B. oviforme	31B	*O. similis*	1	NW Med. Sea	—	JX473680
B. oviforme	33B	*O. similis*	1	NW Med. Sea	—	JX473681

(Continued)

Table A1 | Continued.

Species	Isolate	Host	Number of *Blastodinium* ind. per host	Sampling site	SSU	ITS
B. pruvoti						
B. pruvoti	GA50	*Clausocalanus* sp.	5	Atlantic Ocean	—	JX473682
B. pruvoti	GA51	*Clausocalanus* sp.	5	Atlantic Ocean	FJ541189	—
B. pruvoti	BP01	*Clausocalanus* sp.	2	NW Med. Sea	—	JX473683
B. spinulosum						
B. spinulosum	AS16	*P. parvus*	nd	NW Med. Sea	HQ226070	JX473700
B. spinulosum	BCL04	*Clausocalanus* sp.	10	NW Med. Sea	HQ226071	—
B. spinulosum	BSP02	*Clausocalanus* sp.	20	NW Med. Sea	HQ226072	—
B. spinulosum	BOUM35	Not determined	1	Med. Sea	JN257671	JX473699
B. cf. *spinulosum*	BCL01	*Clausocalanus* sp.	9	NW Med. Sea	JX473663	—
B. spinulosum	GA41	*P. parvus*	13	Atlantic Ocean	—	JX473684
B. spinulosum	BCL03	*Clausocalanus* sp.	14	NW Med. Sea	—	JX473685
***BLASTODINIUM* SP.**						
Blastodinium sp.	SCM16C3	Env. sequence		Sargasso Sea	AY664985	—
Blastodinium sp.	SCM38C54	Env. sequence		Sargasso Sea	AY664986	—
Blastodinium sp.	SCM38C31	Env. sequence		Sargasso Sea	AY664984	—
Blastodinium sp.	SCM16C36	Env. sequence		Sargasso Sea	AY664982	—
Blastodinium sp.	ZZ0053180	Env. sequence		Florida Straits	EU818565	—
Blastodinium sp.	ZZ0053167	Env. sequence		Florida Straits	EU818553	—
Blastodinium sp.	BOUMD9	*Farranula* cf. *rostrata*	2	Med. Sea	JN257679	JX473703
Blastodinium sp.	BOUM29	Not determined	nd	Med. Sea	JN257672	JX473704
Blastodinium sp.	BOUM3	Not determined	nd	Med. Sea	JN257673	JX473705
Blastodinium sp.	BOUM4	*Farranula rostrata*	nd	Med. Sea	JN257678	JX473706
Blastodinium sp.	BOUM7	Not determined	nd	Med. Sea	JN257681	—
Blastodinium sp.	BOUM21	Not determined	nd	Med. Sea	JN257667	JX473707
Blastodinium sp.	BOUM27	Not determined	nd	Med. Sea	JN257668	JX473708
Blastodinium sp.	BOUMF5	*Farranula rostrata*	3	Med. Sea	JN257669	JX473709
Blastodinium sp.	BOUM PARE4	*Paracalanus* sp.	11	Med. Sea	JN257670	JX473710
Blastodinium sp.	BOUMB12	*Oncaea* sp.	1	Med. Sea	JN257675	JX473711
Blastodinium sp.	BOUM11	Not determined	nd	Med. Sea	—	JX473712
Blastodinium sp.	VIL44	*Corycaeus* sp.	nd	Med. Sea	—	JX473713
Blastodinium sp.	VIL48	*Paracalanus parvus*	2	Med. Sea	—	JX473714
Blastodinium sp.	GA90	*Acartia* sp.	1	Atlantic Ocean	—	JX473686

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Microenvironmental ecology of the chlorophyll *b*-containing symbiotic cyanobacterium *Prochloron* in the didemnid ascidian *Lissoclinum patella*

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The discovery of the cyanobacterium *Prochloron* was the first finding of a bacterial oxyphototroph with chlorophyll (Chl) *b*, in addition to Chl *a*. It was first described as *Prochloron didemni* but a number of clades have since been described. *Prochloron* is a conspicuously large (7–25 μm) unicellular cyanobacterium living in a symbiotic relationship, primarily with (sub-) tropical didemnid ascidians; it has resisted numerous cultivation attempts and appears truly obligatory symbiotic. Recently, a *Prochloron* draft genome was published, revealing no lack of metabolic genes that could explain the apparent inability to reproduce and sustain photosynthesis in a free-living stage. Possibly, the unsuccessful cultivation is partly due to a lack of knowledge about the microenvironmental conditions and ecophysiology of *Prochloron* in its natural habitat. We used microsensors, variable chlorophyll fluorescence imaging and imaging of O_2 and pH to obtain a detailed insight to the microenvironmental ecology and photobiology of *Prochloron in hospite* in the didemnid ascidian *Lissoclinum patella*. The microenvironment within ascidians is characterized by steep gradients of light and chemical parameters that change rapidly with varying irradiances. The interior zone of the ascidians harboring *Prochloron* thus became anoxic and acidic within a few minutes of darkness, while the same zone exhibited O_2 super-saturation and strongly alkaline pH after a few minutes of illumination. Photosynthesis showed lack of photoinhibition even at high irradiances equivalent to full sunlight, and photosynthesis recovered rapidly after periods of anoxia. We discuss these new insights on the ecological niche of *Prochloron* and possible interactions with its host and other microbes in light of its recently published genome and a recent study of the overall microbial diversity and metagenome of *L. patella*.

Keywords: *Prochloron*, symbiosis, microenvironment, photobiology, microsensor, bioimaging, didemnid ascidian, *Lissoclinum patella*

INTRODUCTION

The phylum Cyanobacteria harbors a diversity of morphotypes ranging from minute <1 μm unicellular forms (*Prochlorococcus* spp.) up to 100 μm thick filaments (*Oscillatoria* spp.) and large colonial cell aggregations visible by the naked eye (Palinska, 2008). Cyanobacteria are the only oxyphototrophs among prokaryotes and are considered inventors of oxygenic photosynthesis >2.5 billion years ago, major drivers in the formation of the first biological communities in the fossil record, i.e., microbial mats and stromatolites, as well as key architects of the present biosphere through their photosynthetic O_2 production leading to an oxic atmosphere over geologic time scales. Cyanobacteria are also the only oxyphototrophs capable of N_2 fixation, either in specialized cells (heterocysts) or via spatio-temporal modulation of their metabolic activity (Stal and Zehr, 2008), a trait that is employed by several protists, plants, and animals harboring symbiotic cyanobacteria (Adams, 2000; Lesser et al., 2004). In recent years, the analysis of cyanobacterial genomes has revealed a large degree of genetic exchange and plasticity inside this phylum (Zhaxybayeva et al., 2006), and cyanobacteria are regarded as prime candidates involved in the endosymbiosis leading to the evolution of algae and higher plants (Price et al., 2012).

Cyanobacteria also harbor species exhibiting unique characteristics very different from "typical" cyanobacteria. Most notably, this includes the prochlorophytes and the Chl *d*-containing cyanobacteria in the genus *Acaryochloris* that exhibit fundamental differences in terms of pigmentation, structure, and properties of their photosynthetic apparatus (Partensky and Garczarek, 2003; Larkum and Kühl, 2005; Kühl et al., 2007a). More recently, findings of the new chlorophyll *f* in cyanobacterial enrichments from

stromatolites (Chen et al., 2010), and a widespread but yet uncultivated diazotrophic cyanobacterium without a functional PSII (Bothe et al., 2010) add on to the concept of cyanobacteria being a genetic melting pot and origin for a wide variety of photosynthetic adaptations (Larsson et al., 2011; Schliep et al., 2012). The ecology of such exotic cyanobacteria is not well understood, and in this study we focus on the ecology and habitat characteristics of the conspicuous symbiotic cyanobacterium *Prochloron* spp., which can be found and harvested in large quantities from their tunicate hosts (so-called didemnid ascidians) on coral reefs and mangrove systems but has resisted all cultivation attempts since its discovery in 1975 (Lewin and Cheng, 1989).

The taxonomy of *Prochloron* remains ambiguous. The type species was initially called *Synechocystis didemni* and later renamed *Prochloron didemni* (Lewin, 1977). Detailed comparative electron microscopy of different symbiont-host associations indicated several characteristic morphotypes hypothesized to represent different phylotypes of *Prochloron* (Cox, 1986). However, molecular data comparing sequences of the *Prochloron 16S rRNA* gene obtained from different didemnid ascidians and geographic location indicate a fairly strong global similarity (Münchhoff et al., 2007) and a genomic study of *Prochloron* samples along a >5000 km transect in the Pacific showed >97% identity and strong synteny of *Prochloron* genomes (Donia et al., 2011a,b).

Cyanobacteria in the genus *Prochloron* are ∼7–25 µm wide (Cox, 1986), bright green spherical cells with stacked thylakoids oppressed to the cell periphery (**Figure 1**); they were discovered in close association with (sub)tropical didemnid ascidians (Lewin and Cheng, 1975; Newcomb and Pugh, 1975). While the presence of microbial phototrophs and O_2 production in didemnid ascidians was well known (Maurice, 1888; Smith, 1935; Tokioka, 1942), it was a surprise that *Prochloron* contained no phycobilins but Chl *b* in addition to Chl *a* (Lewin and Withers, 1975). At that time, Chl *b* was only known from algae and higher plants, and *Prochloron* was for some time regarded a missing link in chloroplast evolution. However, molecular phylogenetic studies have unequivocally shown that *Prochloron* is but one of several lineages in the cyanobacteria, wherein Chl *b* has evolved (Palenik and Haselkorn, 1992; La Roche et al., 1996).

Along with the other two groups of prochlorophytes, i.e., *Prochlorococcus* and *Prochlorothrix*, *Prochloron* shares several additional differences to other cyanobacteria, including special membrane-bound Chl *a*/*b* light harvesting complexes that are unrelated to the Chl *a*/*b* antenna in the light harvesting complex

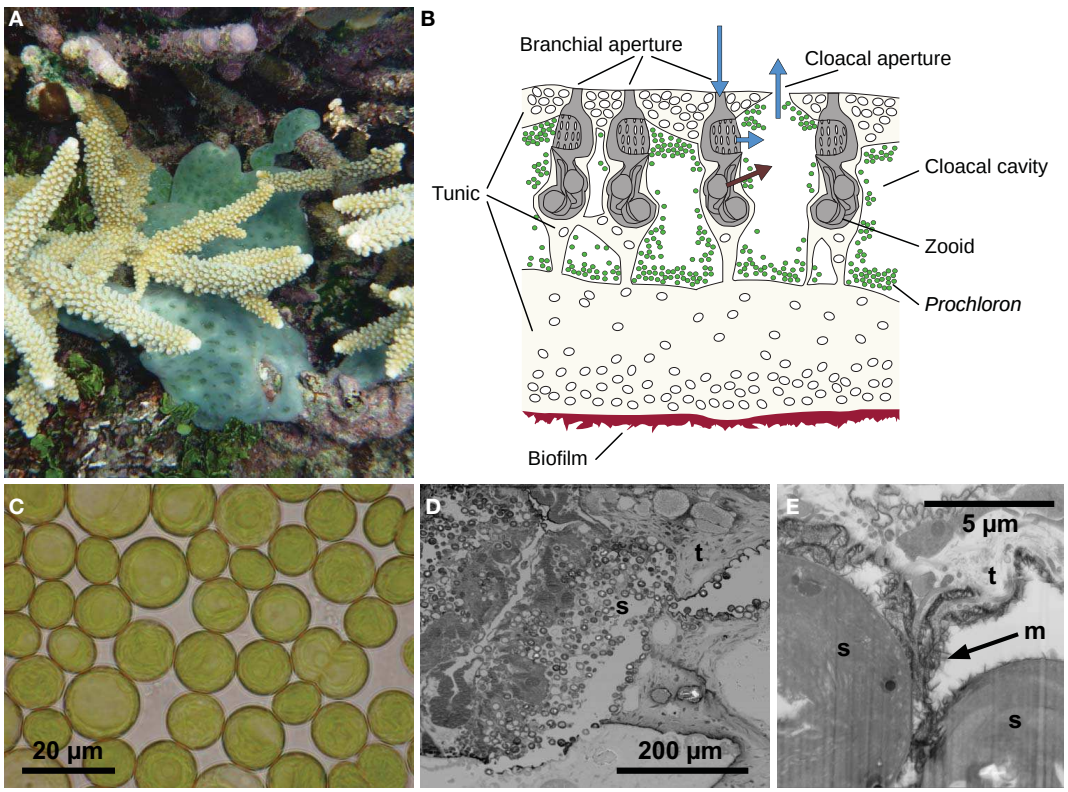

FIGURE 1 | The *Prochloron-Lissoclinum patella* symbiotic association. (A) A colony (∼20 cm^2) of the didemnid ascidian *Lissoclinum patella* covering corals with its 5–10 mm thick opaque cartilaginous tunic, i.e., a matrix of protein and cellulose-like carbohydrates containing calcareous spicules. **(B)** A schematic drawing of a cross-section illustrating the organization of zoids and symbionts in the tunic. The zooids are embedded in the tunic, where they suck in and filtrate particles out of the seawater. Waste products and filtered water are excreted into the surrounding peribranchial space and cloacal cavities in the colony tunic and eventually expelled via a joint cloacal aperture. **(C)** Individual *Prochloron* cells extracted from the ascidians, note the peripheral arrangement of thylakoids in the cells. **(D,E)** Electron micrographs **(D)** [TEM, **(E)** FIB SEM] of the symbiont-host interface in *L. patella* showing round *Prochloron* cells (s) embedded into the host tunic (t) and a fibrous exopolymeric substance (m, arrow). **(C)** Was redrawn and modified after Maruyama et al. (2003).

of eukaryotic oxyphototrophs (La Roche et al., 1996; Partensky and Garczarek, 2003). Interestingly, genes encoding for the so-called "prochlorophyte Chl *b*-binding" proteins (*Pcbs*) are found both in prochlorophytes and in the Chl *d*-containing cyanobacterium *Acaryochloris marina*, which is not closely related to the prochlorophytes on basis of their *16S rRNA* gene phylogeny, indicating a more ancient origin of these genes or lateral gene transfer (Chen et al., 2005).

Prochloron forms symbiotic associations with didemnid ascidians in the genera *Didemnum*, *Tridemnum*, *Lissoclinum*, and *Diplosoma* and represents the only known obligate photosymbiosis in the phylum Chordata (Hirose et al., 2009a). Studies of the *18S rRNA*-based molecular phylogeny of didemnid ascidians indicate that photosymbiosis with *Prochloron* has occurred independently in each of these genera (Yokobori et al., 2006). Besides the unsuccessful attempts to cultivate *Prochloron* separated from its hosts, a symbiotic relation is supported, e.g., by observations of carbon and nitrogen exchange between *Prochloron* and host (Lewin and Pardy, 1981; Kremer et al., 1982; Griffiths and Thinh, 1983; Koike et al., 1993), light-enhanced growth of didemnids with *Prochloron* (Olson, 1986), and fascinating specializations in didemnids ensuring vertical transmission of *Prochloron* to ascidian larvae during maturation and before they leave the parent tunic (Hirose, 2009), i.e., the extracellular matrix of protein and cellulose-like carbohydrates, wherein the host zoids are embedded. However, the exact nature of the symbiosis and mutual benefits in the ascidian-*Prochloron* association is still poorly understood (Hirose and Maruyama, 2004).

Different modes of association between *Prochloron* and didemnid ascidians have been described (Cox, 1986; Hirose et al., 2009a) including (i) colonization of the outer surface and upper tunic, (ii) colonization of the inner cloacal cavities and the peribranchial space of zoids, (iii) a more wide spread colonization in the tunic, and (iv) intracellular *Prochloron* in some didemnid species. Additionally, there is macro- and microscopic evidence that *Prochloron* can be associated with the surface of holothurians (Cheng and Lewin, 1984), sponges (Parry, 1986), and a range of non-didemnid ascidians (Cox, 1986). Significant amounts of suspended *Prochloron* in seawater have been observed (Cox, 1986) and molecular surveys indicated that *Prochloron* could be thriving in stromatolites (Burns et al., 2004), but confirmation of such potentially free-living *Prochloron* awaits further direct evidence of their actual presence and ecological niche in such systems.

Detailed physiological and photobiological studies of *Prochloron* are few (reviewed in Kühl and Larkum, 2002) and have mostly involved measurements on solute exchange or photosynthetic performance of intact ascidian-*Prochloron* associations or on extracted *Prochloron* cells, which only remain photosynthetically competent for a few hours to a day (Critchley and Andrews, 1984). This has hampered both cultivation attempts and a more detailed understanding of the ecological niche of *Prochloron in hospite*. However, some niche characteristics are well known such as the presence of UV absorbing mycosporin-like compounds that act as sunscreens (Dionisio-Sese et al., 1997; Maruyama et al., 2003; Hirose et al., 2006), the presence of enzymes for detoxification of reactive oxygen species (ROS; Lesser and Stochaij, 1990), and the prevalence of a diversity of highly bioactive secondary metabolites (especially cyanobactins) in *Prochloron* that may, e.g., have allelopathic functions against other phototrophs (Schmidt and Donia, 2010; Donia et al., 2011b; Schmidt et al., 2012).

The photosynthetic apparatus of *Prochloron* appears similar to other cyanobacteria with conventional electron transport reactions through photosystem (PS) I and II and a typical oxygen evolution complex. However, special light harvesting supercomplexes based on the prochlorophyte chlorophyll *a/b* (pcb) protein are present in both PSI and PSII (Bibby et al., 2003; Murray et al., 2006). These light harvesting systems give rise to a highly efficient photosynthetic electron transport system, where the maximum quantum yield of PSII approaches 0.82, similar to that in higher plants and much higher than in most cyanobacteria (Schreiber et al., 1997, 2002).

Carboxysomes are present, and a significant part of the carbonic anhydrase present is found in these bodies (Griffiths, 2006) along with all the Rubisco enzyme (Swift and Leser, 1989), which is responsible for fixing CO_2 to phosphoglyceric acid. There appears to be an active supply of organic products to the ascidian host and these appear to be early carbon products of the Calvin–Benson cycle (Kremer et al., 1982). This translocation is host-dependent and represents about 12–56% of reduced carbon for host respiration (Olson and Porter, 1985; Alberte et al., 1987).

Other aspects of metabolism, e.g., the source and dynamics of N_2 fixation and nitrogen turnover in the ascidian-*Prochloron* symbiosis are still unresolved and debated (e.g., Odintsov, 1991; Donia et al., 2011b). There is evidence that in addition to carbon, nitrogen is also recycled in the symbiosis (Koike et al., 1993). Ammonium is the major nitrogenous waste of the host (Goodbody, 1974) and is taken up by *Prochloron* (Parry, 1985). It has been proposed that N_2 fixation contributes to the nitrogen requirements (Paerl, 1984). This was disputed by Parry (1985), but is supported by nitrogen isotope ratios in host and *Prochloron*, which are consistent with nitrogenase activity (Kline and Lewin, 1999).

The recent publications of the first draft *Prochloron* genome as well as studies of the microbiome of photosymbiotic didemnids (Donia et al., 2011a,b; Behrendt et al., 2012a) have now added significant new insights to the ecology of *Prochloron*, and potential interactions with its didemnid hosts and other members of the microbiome. However, the interpretation of such molecular surveys in terms of physiology and metabolic interactions mostly rely on genomic evidence that still needs experimental verification for *Prochloron* under natural conditions.

While many studies have speculated about the microenvironmental conditions of *Prochloron in hospite*, very few experimental data on the microenvironment in didemnid ascidians have been published (Kühl and Larkum, 2002; Behrendt et al., 2012a). These data indicate highly dynamic physico-chemical conditions that are strongly modulated by irradiance. In this study, we present new data and review the current knowledge about the microenvironment and metabolic activity of symbiotic *Prochloron* associated with didemnid ascidians. We focus on the large colonial didemnid ascidian *Lissoclinum patella*, which covers significant areas of substratum on coral reefs with an opaque cm-thick cartilaginous tunic harboring large quantities of *Prochloron* in the internal peribranchial and cloacal cavities of the filter-feeding zoids (**Figure 1**). The tunic of *L. patella* is an integumentary extracellular matrix of protein and cellulose-like carbohydrates covered

by a dense tunic surface cuticle containing calcareous spicules as well as a diversity of specialized tunic host cells (Goodbody, 1974; Hirose, 2009).

Using microsensors and advanced bioimaging, we present detailed data on the physical and chemical boundary conditions *in hospite* and show how the metabolic activity of *Prochloron* and its host modulate the microenvironmental conditions in response to changes in irradiance. We discuss these new insights into the ecological niche of *Prochloron* and possible interactions with its host and other microbes in light of its recently published genome and recent studies of the overall microbial diversity and metagenome of the didemnid ascidian host *L. patella*. (Donia et al., 2011a,b; Behrendt et al., 2012a).

RESULTS

DISTRIBUTION AND PHOTOSYNTHETIC ACTIVITY OF *PROCHLORON*

Both macroscopic imaging and electron microscopy of *L. patella* sections showed a dense colonization of the cloacal cavities and the peribranchial space of zooids by apparently intact and dividing *Prochloron* cells (**Figures 1** and **2A**). *Prochloron* was closely associated to the host tunic, either directly embedded in the tunic or anchored in an exopolymeric substance apparent in the electron micrographs (**Figures 1D,E**).

High absorptivity of red light was observed in the densely pigmented *Prochloron* layer and the cyanobacterial biofilm underneath the ascidian, while more faint light absorption was observed associated with the tunic matrix especially at the colony surface and immediately below the *Prochloron* layer (**Figure 2B**). *Prochloron* exhibited a high maximum PSII quantum yield of ∼0.8 after dark acclimation *in hospite*, while the effective PSII quantum yield declined down to ∼0.2 with increasing levels of incident irradiance (**Figure 2C**). The cyanobacterial biofilm exhibited a lower maximum PSII quantum yield of ∼0.5–0.6 decreasing to an effective PSII quantum yield of <0.1 at the highest irradiance. Using the product of absorptivity, quantum yield and incident irradiance as a proxy for photosynthetic activity, the *Prochloron* and cyanobacterial biofilm also showed different acclimation to irradiance (**Figure 2D**), while more faint activity was found in a thin layer on the tunic surface and a layer immediately below the *Prochloron* cells. The cyanobacterial biofilm exhibited onset of saturation, as determined from the intersection of the initial slope and the maximal activity level, at an irradiance of ∼100 μmol photons m^{-2} s^{-1}, while the *Prochloron* layer showed saturation of photosynthesis at irradiances >250 μmol photons m^{-2} s^{-1}. Both zones exhibited no photoinhibition due to short exposure to irradiances of ∼1000 μmol photons m^{-2} s^{-1}. Imaging of the effective quantum yield of PSII and the derived relative photosynthesis revealed further details on the distribution of photosynthetic activity in *L. patella* (**Figures 2E–L**). The activity distribution in the cloacal and peribranchial parts of *L. patella* closely followed the distribution of the green colored areas indicative of *Prochloron* and the pink-colored cyanobacterial biofilm (compare, e.g., **Figures 2A,I**).

LIGHT PENETRATION AND ATTENUATION IN *L. PATELLA*

Scalar irradiance measurements in intact *L. patella* specimen revealed both a pronounced scattering of incident light in the upper tunic as well as strong attenuation of visible light (VIS; 400–700 nm) in the deeper layers (**Figure 3A**). VIS was strongly scattered in the uppermost tunic leading to a local increase of scalar irradiance reaching ∼140–170% of incident light. About 10–40% of the incident VIS penetrated the upper tunic, while strong attenuation in the *Prochloron*-containing layer (ranging in depth from ∼1.5–2 to ∼4–6 mm below the tunic surface) reduced VIS down to only a few % of the incident irradiance (**Figure 3A**). In the lower part of the tunic, attenuation of VIS was less strong.

Near-infrared radiation (NIR) showed much less attenuation but strong scattering in *L. patella* (**Figure 3A**). The scalar irradiance of NIR (700–750 nm) reached ∼170–230% of downwelling NIR in the uppermost tunic. About 30–40% of the incident NIR still prevailed below the *Prochloron* layer and NIR was less strongly attenuated in the lower part of the tunic.

The scalar irradiance spectra showed further details on the propagation and attenuation of light in *L. patella* (**Figure 3B**). In the NIR region, weak minima around 800–805 and 870–880 nm indicated the presence of some BChl *a*, while a stronger minimum at 965–985 nm may indicate the presence of another BChl-like photopigment. However, NIR was much less attenuated than visible wavelengths and combined with strong scattering this led to significant local enhancement of the scalar irradiance in the uppermost tunic.

In the visible spectral region, scalar irradiance showed strong spectral minima corresponding to absorption maxima of several photopigments: a strong minimum at 675 nm and a shoulder at ∼650 nm revealed the presence of Chl *a* and Chl *b* indicative of *Prochloron*: a minimum at ∼625 nm, as well as minima at 585–590, ∼565, and a shoulder at ∼490–495 nm indicated presence of phycobiliproteins in *L. patella*. In the upper tunic, distinct minima at ∼390 and ∼426 nm could indicate the presence of photoprotective pigments, but we did not confirm the type or quantity of such compounds in this study.

DISTRIBUTION AND DYNAMICS OF O_2

Profiling with thin O_2 microelectrodes revealed a highly dynamic microenvironment of *Prochloron* inside *L. patella*, which was strongly regulated by ambient irradiance levels (**Figure 4**). In the dark, only the uppermost part of the test remained oxic, while the interior of *L. patella* was anoxic down to about 4 mm depth into the ascidian colony, although the surface was exposed to flowing aerated seawater in the flow chamber. Upon exposure to light, the ascidian showed rapid accumulation of O_2 in its interior cavities due to intense photosynthesis in the densely populated *Prochloron* biofilm lining the peribranchial space and the internal cloacal cavities, while the upper tunic exhibited O_2 consumption both in darkness and light (**Figure 4A**). Under an irradiance of 93 μmol photons m^{-2} s^{-1}, O_2 started to accumulate ∼2 mm below the tunic surface, where the microsensor entered the *Prochloron* layer; further, into this layer the declining O_2 concentration indicated light limitation and net O_2 consumption. At higher irradiance O_2 increased rapidly and approached saturating levels reaching ∼250% air saturation in the *Prochloron* layer at irradiances >300 μmol photon m^{-2} s^{-1}.

The O_2 dynamics inside the ascidian was strongly affected by irradiance. Experimental light-dark shifts with the O_2 microsensors fixed ∼3 mm below the ascidian surface in the *Prochloron* layer

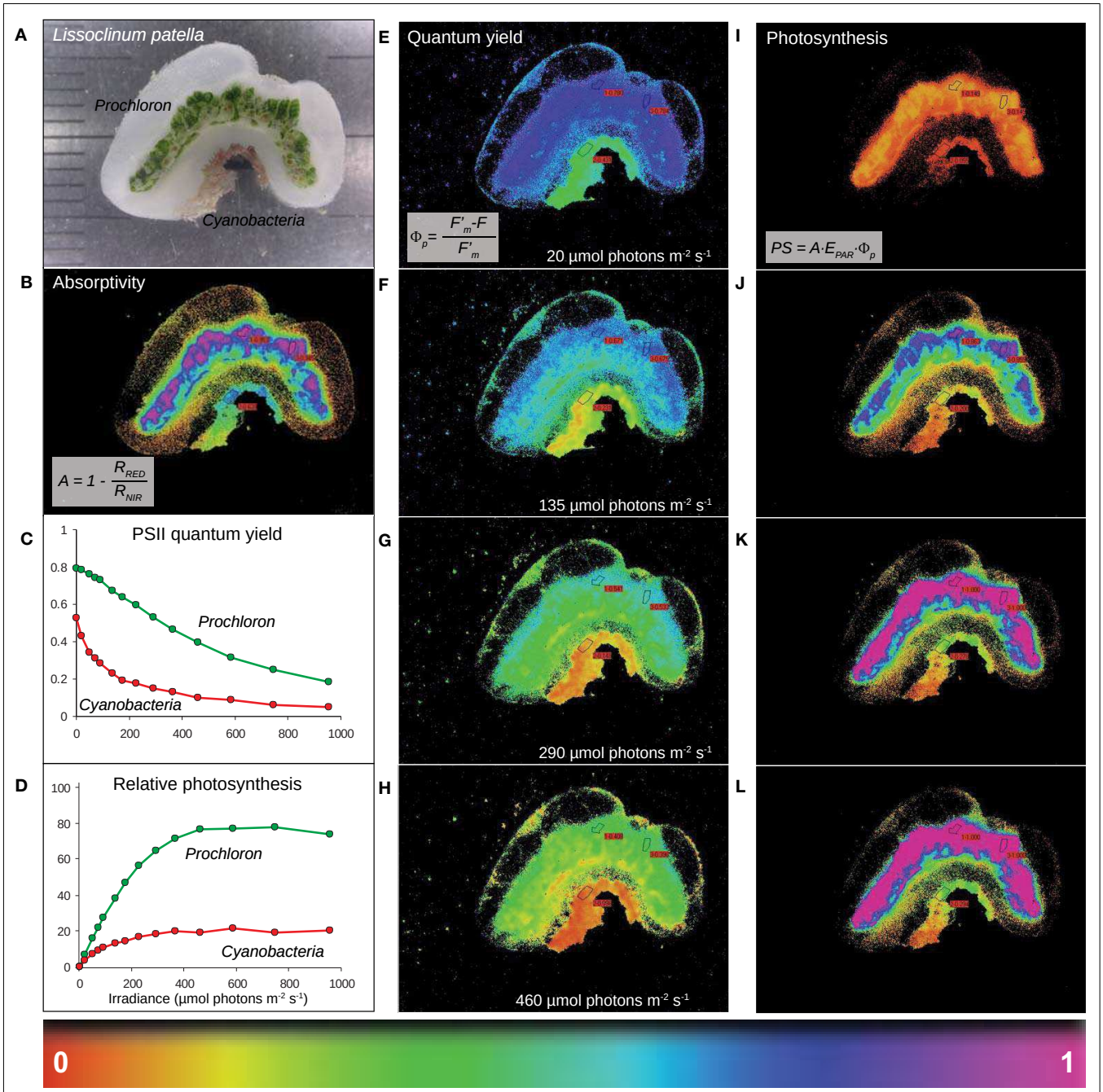

FIGURE 2 | Imaging of light absorption and relative photosynthetic activity of *Prochloron in hospite*. **(A)** A cross-section of *L. patella* showing a bright green layer of *Prochloron* cells in the cloacal cavities and the peribranchial space of the zooids and a reddish cyanobacterial biofilm colonizing the underside of the basal tunic. Light scattering spicules are embedded in the tunic, especially in the upper tunic and immediately below the *Prochloron* layer. **(B)** Absorptivity of red light (λ_{max} 650 nm) in the different layers of *L. patella*. **(C,D)** Quantum yield of PSII and derived relative photosynthesis vs. irradiance curves for regions of interest (ROI) in the *Prochloron* layer and the cyanobacterial biofilm colonizing the underside of the ascidian. **(E–H)** Spatial distribution of PS II quantum yield and the derived relative photosynthesis **(I–L)** in *L. patella* at selected incident irradiance levels, E_{PAR}. A proxy for the relative photosynthetic activity was calculated from the relative PSII electron transport rate $rETR = \Phi_{PSII} E_{PAR}$, taking the different absorptivity over the imaged specimen into account, as $PS = A \Phi_{PSII} E_{PAR}$.

showed alternation from anoxic to hyperoxic conditions over time scales ranging from 15 to 30 min, where the rate of O_2 build-up increased with irradiance indicative of an increased photosynthetic rate (**Figure 4B**). Even after prolonged time in darkness and anoxia, *Prochloron* photosynthesis exhibited an immediate onset of O_2 production upon illumination. However, during profiling

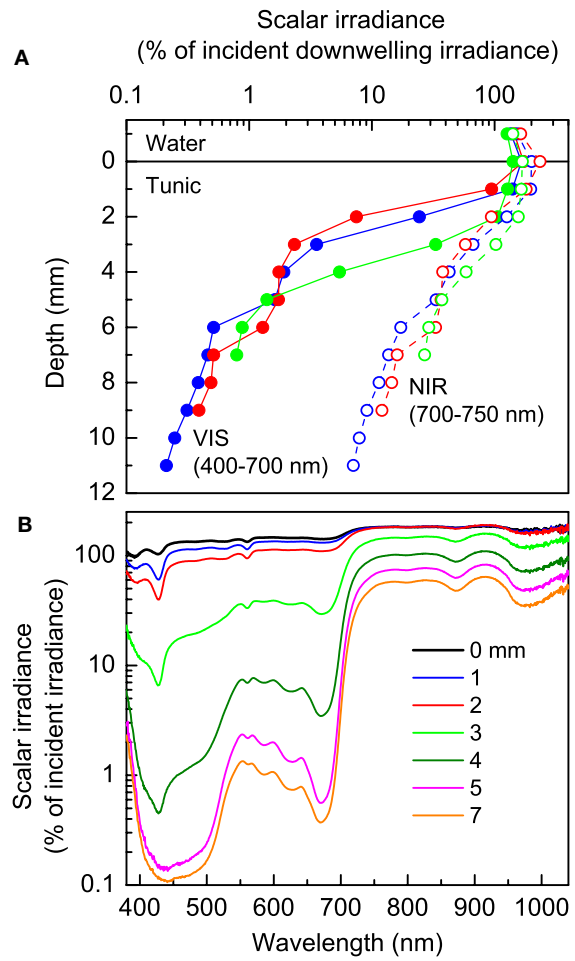

FIGURE 3 | Light propagation in *L. patella*. (A) Depth profiles of integrated scalar irradiance (in % of the incident downwelling irradiance at the tunic surface) for VIS (VIS, 400–700 nm) and the part of near-infrared radiation (NIR, 700–750 nm) which can potentially drive oxygenic photosynthesis via Chl *d* or *f*. **(B)** Spectral scalar irradiance (in % of the downwelling spectral irradiance at the tunic surface) measured at increasing depth below the tunic surface (given as numbers on curves).

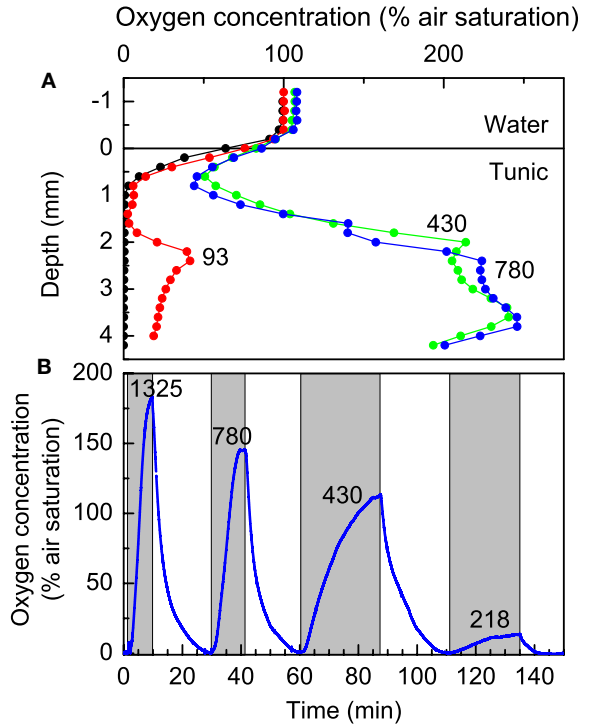

FIGURE 4 | Oxygen dynamics in *L. patella*. (A) Depth profiles of O_2 concentration (in % air saturation) at increasing irradiance levels (given as numbers on curves in units of μmol photons m^{-2} s^{-1}). **(B)** Dynamics of O_2 concentration at ~3 mm depth in a *L. patella* specimen under experimental light-dark shifts at different irradiance levels (given as numbers on curves in units of μmol photons m^{-2} s^{-1}). Shaded areas indicate periods of illumination.

we often observed that the O_2 level in the uppermost tunic of *L. patella* decreased over time upon repeated profiling, especially under high irradiance (data not shown), and such apparent wound reactions caused long term downward drift in many measurements and limited the amount of O_2 profiles obtained.

While the O_2 microsensors only provided a limited number of spot measurements in the ascidians (**Figure 4**), the O_2 dynamics were confirmed by imaging of O_2 concentration across a *L. patella* specimen sliced vertically and pressed up against an O_2 sensitive foil mounted inside the transparent flow chamber (see Materials and Methods for further details). This O_2 imaging revealed similar dynamics in *L. patella* as observed in the microsensor data, showing anoxia in the *Prochloron* layer in darkness and enhanced O_2 due to onset of photosynthesis upon illumination (**Figure 5**). However, the actinic light source used in the imaging setup only allowed homogenous illumination of the ascidian with a maximal photon irradiance of 250 μmol photons m^{-2} s^{-1} and this did not enable high super saturating O_2 levels to be reached.

Oxygen imaging revealed several zones in the ascidian contributing to the build-up of O_2 (**Figures 5C–E**). Initially and shortly after onset of illumination, O_2 evolution was detected in separate zones comprising the upper tunic surface, the *Prochloron* layer and the cyanobacterial biofilm layer on the ascidian underside (**Figure 5D**). As the O_2 levels continued to increase and oxygenate the ascidian tissue, these distinct zonations between the test surface and the *Prochloron* layer became less distinct, while a second zone of O_2 production just below the *Prochloron* layer and above the cyanobacterial biofilm became visible (**Figures 5F,G**). Upon darkening, the *Prochloron* layer exhibited the fastest O_2 depletion, while the other regions in *L. patella* approached anoxia more gradually (**Figures 5I–L**). The upper tunic and the biofilm on the underside of *L. patella* remained oxic in darkness, albeit at low levels of ~10–20% air saturation (**Figure 5**; ROI 1 and 6 in **Figure 6**).

DISTRIBUTION AND DYNAMICS OF PH

pH is another chemical parameter in the ascidian host that potentially is strongly affected by *Prochloron* photosynthesis due to their CO_2-fixation. Attempts to profile pH with glass needle microelectrodes were not successful due to their fragility and apparent local release of acid vacuoles in the ascidian tunic when inserting the

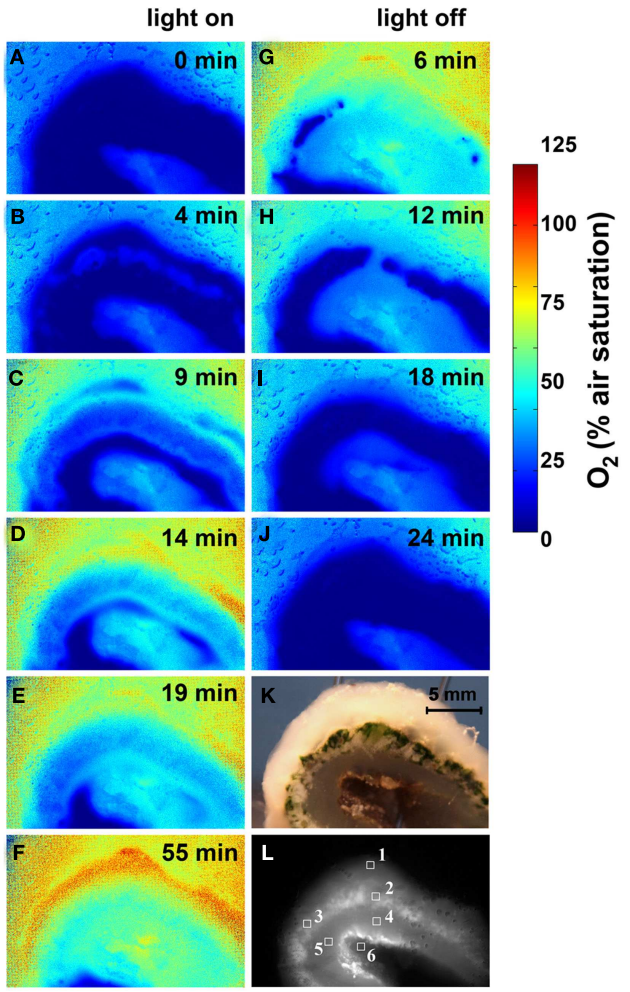

FIGURE 5 | Imaging of O_2 concentration dynamics in *L. patella*. (A–J) Pseudocolor images of the O_2 distribution in *L. patella* under an incident photon irradiance of 250 μmol photons m^{-2} s^{-1} and after darkening. Times in light and after darkening are indicated in minutes in each panel. **(K)** Photograph of *L. patella* cross-section. **(L)** Image of cross-section illuminated with red light showing the *Prochloron* layer and the biofilm underneath the ascidian. Regions of interest (ROI) show the origin of data plotted in **Figure 6**.

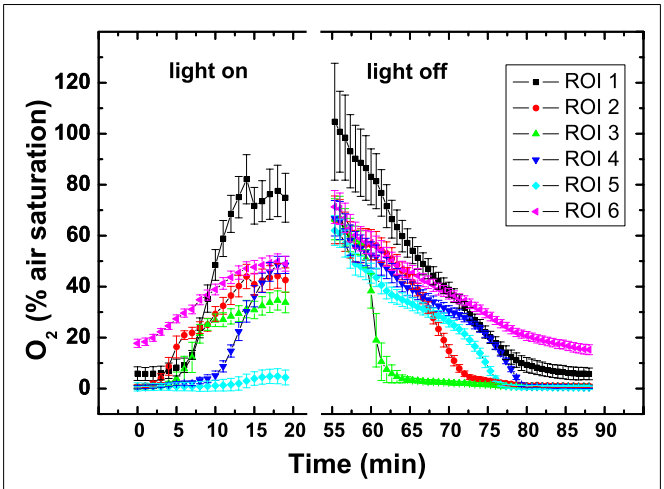

FIGURE 6 | Dynamics of O_2 concentration in selected regions of interest (ROI; see Figure 5L) after on set of illumination with a photon irradiance of 250 μmol photons m^{-2} s^{-1} and after darkening. Symbols represent the average ± standard deviation in each ROI.

relatively large needle electrodes (data not shown). Therefore, we adapted a new pH imaging approach to resolve the pH dynamics in *L. patella*. As this is the first time such imaging has been applied, detailed data on sensor performance, and calibration are given in the methods section at the end of this article.

The pH dynamics in *L. patella* showed pronounced spatio-temporal pH variations and distinct differences to the pH in the surrounding seawater (**Figures 7** and **8**). The layers containing *Prochloron* in the peribranchial space and common cloacal cavity (e.g., ROI 1 in **Figure 7**, monochrome image) showed the most pronounced pH dynamics with irradiance, shifting from about pH 7 to 7.5 in darkness to almost pH 10 under an irradiance of 250 μmol photons m^{-2} s^{-1}. This shift occurred within ∼20 min in response to a light-dark shift.

Layers in the upper tunic (e.g., ROI2 in **Figure 7**, monochrome image) and the basal tunic (e.g., ROI3 and ROI5 in **Figure 7**, monochrome image) showed slightly acidic conditions around pH 6.5–7 both in darkness and during illumination. A patch of cyanobacterial biofilm colonizing the underside of *L. patella* (ROI4 in **Figure 7**, monochrome image) showed a slightly higher pH reaching pH 7.5–7.8 (with the exception of one time point showing an intermittent dip to pH 7.0), and exhibited no clear light-dark dynamics under the experimental irradiance levels.

The pH images showed some conspicuous hot spots of strong pH dynamics in part reflecting different amounts of *Prochloron* in the cloacal cavities (**Figure 7**). However, the surrounding host tissue also exhibited an interesting pattern of fluctuating pH, e.g., initially becoming more acidic upon onset of illumination and then exhibiting fluctuating yet acidic pH conditions (**Figure 7**, light on 0–60 min). Such fluctuations and hot spots were also evident after darkening, where the host tissue in some regions of the upper test apparently became less acidic (**Figure 7**, light off 10–30 min).

DISCUSSION

Application of microsensors and bioimaging revealed new and, up to now, the most detailed insights to the microenvironment of *Prochloron* and its host *L. patella* and confirmed preliminary data obtained in the didemnid ascidian *Diplosoma virens* (Kühl and Larkum, 2002) and the first few light and O_2 measurements in *L. patella* obtained during a survey of its microbiome (Behrendt et al., 2012a). In addition to more detailed microsensor measurements of O_2 and scalar irradiance, this study presents the first use of O_2 and pH imaging in photosymbiotic ascidians and combines this new methodology with variable chlorophyll fluorescence imaging of light absorption and PSII activity at similar high spatio-temporal resolution. This allowed us to map both the dynamic chemical landscape and the distribution of photosynthetic activity onto the structural heterogeneity of *L. patella*

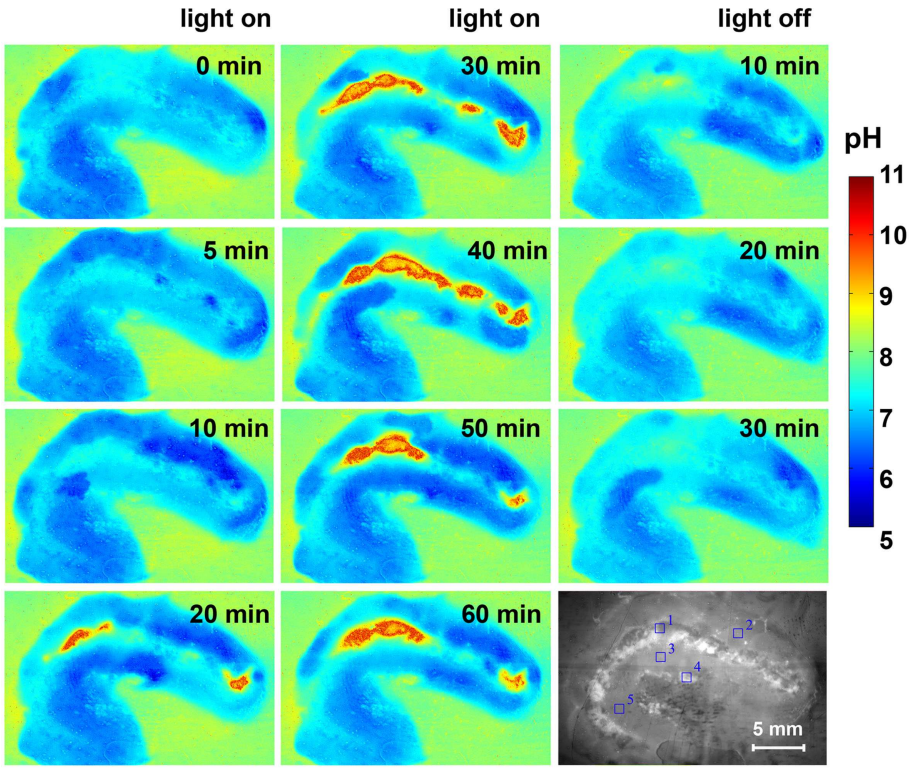

FIGURE 7 | Pseudocolor images of the pH distribution in *L. patella* under an incident photon irradiance of 250 μmol photons m^{-2} s^{-1} and after darkening. Times in light and after darkening are indicated in minutes in each panel. The monochrome image (lowermost right panel) is the cross-section illuminated with red light showing the *Prochloron* layer and the biofilm underneath the ascidian. Regions of interest (ROI) are outline din blue and show the origin of data plotted in **Figure 8**.

and its photosymbionts. In the following, we discuss the implications of these new microenvironmental analyses in light of what is currently known about the physiology and diversity of photosymbionts in the *Prochloron*-didemnid ascidian association and its associated microbiome.

OPTICAL PROPERTIES AND LIGHT PROPAGATION

Microscale light measurement showed that both visible and NIR were scattered strongly in the opaque tunic of *L. patella* and such local photon trapping led to local maxima in scalar irradiance significantly above the incident downwelling irradiance, i.e., up to 170 and 230% for VIS and NIR, respectively. Similar photon trapping has been observed in different optically dense biological systems with pronounced scattering like sediments, biofilms, plant, and animal tissue (e.g., Vogelmann and Björn, 1986; Vogelmann, 1993; Kühl et al., 1994, 1995; Magnusson et al., 2007). The magnitude of the scalar irradiance maximum is modulated by the optical properties of the system, especially the refractive index, the absorption and scattering coefficients as well as the characteristic scattering phase function of the matrix (see details in Kühl and Jørgensen, 1994).

The optical properties of the tunic matrix are largely unknown but with the exception of the genus *Diplosoma*, photosymbiotic didemnids are known to contain scattering spheroid spicules in their tunic. It has been speculated that these calcareous spicules could have an important role for light propagation and photoadaptation in the tunic of photosymbiotic ascidians (Hirose et al., 2006). A role for spicules in light guiding has been demonstrated

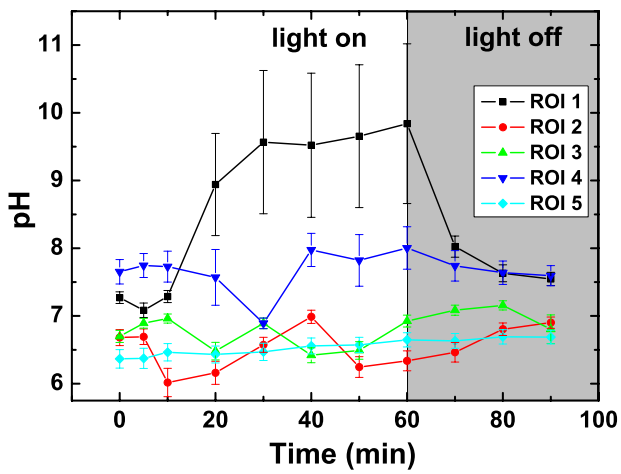

FIGURE 8 | Dynamics of pH in selected regions of interest (ROI; see Figure 7) after on set of illumination with a photon irradiance of 250 μmol photons m^{-2} s^{-1} and after darkening. Symbols represent the average ± standard deviation in each ROI.

in sponges harboring photosymbionts, where radially arranged bundles of spicules channel light into deeper layers of the sponge (Brümmer et al., 2008). We did not see any evidence for light guiding in *L. patella* when imaging cross-sections of a colony, while irradiating it from above. However, it is known from other tissues, such as leaves (Gorton et al., 2010) and corals (Wangpraseurt et al., 2012), that direct and diffuse light can have very different results on light scattering in internal tissues.

Moreover, spicules in photosymbiotic ascidians do not form bundles and can be regarded amorphous scattering particles distributed in the tunic – often at higher density in the upper layers. Hirose et al. (2006) studied the *Prochloron*-containing ascidian *Didemnum molle*, which showed a decreasing density of spicules in the tunic with increasing water depth, and they proposed that high spicule density in shallow water primarily induced photoprotection by increasing the reflectivity of the ascidians. We are not aware of similar studies of the spicule density in *L. patella*, but our light measurements showed pronounced scattering in the upper tunic leading to photon trapping and local amplification of scalar irradiance, especially in the NIR region, where absorption in the tunic is minimal and such scattering effects therefore are most pronounced (**Figure 3**).

Increased photon pathlength due to spicule-induced scattering in the tunic is but one of several mechanisms potentially affecting light levels and photon trapping. The refractive index of the tunic matrix relative to that of the surrounding seawater can also play an important role as refractive index mismatches can enhance redirection of upwelling scattered light back into the tunic and further enhance photon pathlengths of scattered light (Kühl and Jørgensen, 1994).

A somewhat similar mechanism, assuming absence of scattering in the tissue layer, has been proposed in corals, where multiple scattering and enhanced diffuse reflectance from the coral skeleton underneath leads to a more efficient absorption of incident light by photopigments (Enriquez et al., 2005). In the case of completely diffuse backscatter, the average path length of upwelling photons is twice that of photons in a collimated light beam traversing a thin layer of tissue (Kühl and Jørgensen, 1994). This implicates that the probability for a photon to become absorbed is significantly enhanced, as long as the density of absorbers is not so high that self-shadowing and package effects arise. As a result of this phenomenon, corals inhabiting high light environments can maximize their absorption capacity with low pigment investment while reducing self-shading (Enriquez et al., 2005). Similar effects may be at play in the spicule-containing tunic of photosymbiotic ascidians.

All the above mentioned light trapping and scattering mechanisms lead to enhanced spectral filtering as an increased photon path length due to scattering increases the probability for encountering absorbing pigments in the tunic. Accordingly, photons at wavelengths within major absorption bands of host and photosymbiont pigments will be absorbed more efficiently, while photons outside such absorption bands will be unaffected leading to larger relative differences in the light spectrum. This can have important implications for the *Prochloron*-ascidian association, where UV-screening mycosporine like aminoacids (MAAs) have been found in the upper tunic (e.g., Hirose et al., 2004). Genomic analyses show that such compounds can be synthesized by *Prochloron* (Donia et al., 2011b) but the translocation to the ascidian host tissue remains to be studied, and *Prochloron* may not be the only source. We hypothesize that the presence of spicules and the scattering properties of the upper tunic enable efficient UV protection in analogy to similar effects in corals (Enriquez et al., 2005), while at the same time ensuring that sufficient visible wavelengths can propagate into deeper layers with *Prochloron*. Interestingly, we also found a higher density of spicules in the tunic immediately below the *Prochloron* layer, which may further affect the light field and spectral absorption in *L. patella*, but more detailed optical studies are needed to test such hypotheses.

IN VIVO CONDITIONS AND PHOTOSYNTHESIS OF *PROCHLORON*

Our microenvironmental measurements show that *Prochloron*, in the peribranchial space and cloacal cavity, inhabits a very dynamic ecological niche in *L. patella*, wherein chemical conditions respond rapidly to changes in irradiance over a timescale of 15–30 min. In the case of O_2, levels changed quickly from anoxic in the dark up to >250% O_2 air saturation at PAR irradiances >300 μmol photon m^{-2} s^{-1} (**Figure 4B**). The O_2 imaging approach supported these results and in addition allowed a much more detailed assessment of the contributions of other oxygenic photosymbionts in *L. patella*.

The pH imaging system revealed that the pH in the peribranchial/common cloacal cavity shifted from ∼pH 7.0 to pH 7.5 in the dark to just below pH 10.0 under a PAR irradiance of ∼250 μmol photon m^{-2} s^{-1}. The pH in this zone is thus lower than in seawater (∼pH 8.2) in the dark and much higher under moderately high irradiance. As the peribranchial/common cloacal cavities are connected to the external seawater by inhalant siphons, one per branchial cavity, it is clear that the internal environment is strongly influenced by the ascidian tissues and *Prochloron* both in the dark and in the light. While photosynthesis can proceed at >pH 9.5 (e.g., Giordano et al., 2005) it is entirely dependent on HCO_3^- at that pH, since CO_2 is absent under such alkaline conditions. This suggests that inorganic carbon transport in *Prochloron* is mainly HCO_3^--dependent. This conclusion is consistent with the evidence from a recent genomics study (Donia et al., 2011b) showing that *Prochloron* only has low-affinity carbon transport pumps. *Prochloron* also lacks many transporters found in other cyanobacteria, including those involved in high-affinity CO_2 uptake (NdhD3-F3) and the low-CO_2 inducible bicarbonate transporters *sbtA* and *CmpA-D* (Badger et al., 2002). Interestingly, the upper and lower tunic layers that showed some evidence of O_2-evolving cyanobacteria were acidic in the light, possibly reflecting a much greater influence of surrounding ascidian cells. The slightly acidic tissue adjacent to the peribranchial/common cloacal cavities and the local hotspots also reflected changes in the animal host tissues, probably in response to the massive change in pH of the peribranchial/common cloacal cavities.

The data presented here for the first time show *in vivo* photosynthesis of *Prochloron* in the peribranchial/cloacal cavity to be both high and efficient (**Figures 2–4**). Early work on freshly extracted *Prochloron* (Critchley and Andrews, 1984) indicated that photosynthetic O_2 evolution was very efficient

[up to 180 μmol O_2 (mg Chl)$^{-1}$ h^{-1}] under irradiances of 300–400 μmol photons m^{-2} s^{-1}, supported by *in vivo* measurements of variable chlorophyll fluorescence (Schreiber et al., 1997). The direct measurements here are similar (**Figure 2**) and the light measurements suggest that in shallow reef waters *Prochloron* can experience irradiance levels of >300–400 μmol photons m^{-2} s^{-1} *in hospite*. It is noteworthy that in our variable chlorophyll fluorescence imaging, we used blue actinic light exposing a cut surface of *L. patella* evenly, so that the filtering effect of the upper tunic was avoided. The apparent lack of photoinhibition indicates that even at higher irradiance photosynthesis would not be impaired. Our data are in line with earlier observations on high irradiance tolerance of photosynthesis in *Prochloron* when associated with its didemnid host (e.g., Alberte et al., 1987; Lewin and Cheng, 1989). The presence of UVA/B absorbing MAAs in the outer tunic, shown in previous work (e.g., Hirose et al., 2004) and by the *in vivo* spectra here (**Figure 3B**), could be an important factor allowing high photosynthetic rates of *Prochloron in situ* even at high ambient solar irradiance. Furthermore, Lesser and Stochaij (1990) demonstrated the presence of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, and catalase in the *Prochloron-L. patella* symbiosis, and showed that the activity of these enzymes were directly proportional with irradiance. *Prochloron* thus seems well adapted to quench ROS formed in the photosynthetic apparatus under high irradiance. This is further supported by the presence of multiple *hli* genes in the genome of *Prochloron* (Donia et al., 2011b). These genes encode so-called high light induced proteins that are thought to play a role in quenching of excess excitation energy (He et al., 2001).

As pointed out in the above section, under these high photosynthetic conditions, the O_2 concentrations and pH in the peribranchial/cloacal cavity rise to very high levels (**Figures 5–9**). Clearly neither of these factors inhibits the photosynthetic capacity of *Prochloron* and as pointed out above, the low-affinity inorganic carbon transporters in the plasma membrane of *Prochloron* must be able to transport adequate quantities of HCO_3^- into the cells. Whether any role is played by matrix compounds present in the *Prochloron* layer (**Figure 1**), and whether the host exerts any control on the uptake of inorganic carbon across the plasma membrane (Critchley and Andrews, 1984; Griffiths, 2006) are important questions for future research.

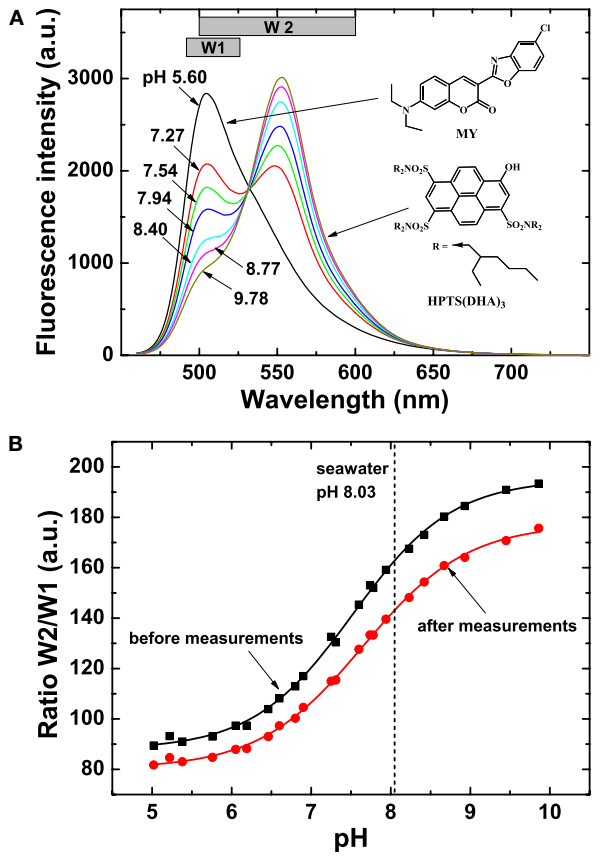

FIGURE 9 | Spectral characteristics and calibration of sensor foils used for pH imaging. (A) Spectral properties of the pH sensor material showing emission spectra of the pH planar optode (λ_{exc} = 450 nm, 0.6 M NaCl, 25°C) at varying pH (indicated on curves), chemical structures of the dyes and the windows (W1 and W2) used for the interrogation with a multispectral imaging system. The spectra were obtained on a Hitachi F-7000 fluorescence spectrometer. **(B)** Calibration curves of the pH sensor foil, i.e., a plot of the ratio of W2/W1 vs. defined pH levels. Note that the optimal dynamic range of the optode is at pH 6–9. The sigmoidal calibration curve flattens at pH >9 and <6, and pH values obtained in these ranges are thus less accurate.

OTHER OXYPHOTOTROPHS IN DIDEMNID ASCIDIANS

Besides *Prochloron*, other phototrophs occur in the upper unic of *L. patella* and as judged from the *in vivo* spectral light measurements (**Figure 3**) appear to be phycobilin-containing cyanobacteria. This is in line with the findings in a recent survey of the microbial diversity in *L. patella*, where *16S RNA* gene sequences and hyperspectral signatures of cyanobacteria were abundant in the upper tunic (Behrendt et al., 2012a). Another cyanobacterial zone also lies in the tunic beneath the *Prochloron* layer, the photosynthetic activity of which became evident in the imaging of PSII activity, O_2 and pH. The slower dynamics of O_2 in this layer is probably the result of light screening by cyanobacteria in the upper test and the *Prochloron* layer. Both zones of cyanobacteria have been observed before and the tunic is known to harbor the cyanobacterium *Synechocystis trididemni* and other cyanobacteria with special pigmentation (Lafargue and Duclaux, 1979; Kott, 1984; Cox et al., 1985; Hirose et al., 2009b; López-Legentil et al., 2011). López-Legentil et al. (2011) found Chl *d*-containing *Acaryochloris cells* in the unic of other didemnid ascidians on mangrove roots in the Bahamas but we have not found any evidence for Chl *d* in the unic of *L. patella* (Kühl et al., 2005; Behrendt et al., 2012a).

The other oxyphototrophs in *L. patella* are exposed to very different irradiance regimes in comparison to *Prochloron*. Those colonizing the surface and upper tunic would be exposed, at times, to high and potentially photoinhibitory irradiance levels reaching >400 μmol photons m^{-2} s^{-1}. In contrast, *S. trididemni* and other cyanobacteria below *Prochloron* layer would be much better shielded and prone to light limitation at high irradiance as a result of the strongly absorbing *Prochloron* layer above (**Figure 3**). Due to strong light attenuation and spectral filtration by *Prochloron*, the cyanobacteria thriving below it have adapted their pigmentation

and *S. trididemni* and other cyanobacteria present contain phycourobilin in addition to other more conventional phycobiliproteins (Cox et al., 1985; Hirose et al., 2009b), which would allow them to harvest green light very efficiently; it is probably phycourobilin that contributes to the high absorption at around 500 nm in the deeper tunic layers (**Figure 3B**; cf Hirose et al., 2009b). In the present study, we did not specifically target the identification and abundance of these cyanobacteria in the tunic of *L. patella*. Behrendt et al. (2012a) also did not extend their microbial diversity survey to the inhabitants of the lower tunic in *L. patella*; however, our data point to a rather dense and active community, which awaits further investigation.

Our imaging studies also revealed the presence of oxyphototrophs in the biofilm layer colonizing the underside of *L. patella*. This biofilm is highly diverse, with cyanobacteria as the predominant oxyphototrophs, especially *A. marina* that thrives in this ecological niche deprived of visible wavelengths but with abundant NIR supporting its Chl *d*-based oxygenic photosynthesis (Kühl et al., 2005; Behrendt et al., 2012a; Larkum et al., 2012). The present results thus confirm our previous studies (Kühl et al., 2005, 2007a) showing active photosynthesis in this biofilm albeit with a more moderate dynamics in both O_2 and pH as compared to *Prochloron*. Actually, both the O_2 and pH imaging data represent the very first *in vivo* measurements of these parameters in the natural habitat of *A. marina*, but a detailed discussion of the implications of these observations for our knowledge on the *in situ* biology of Chl *d*-containing cyanobacteria is beyond the scope of this study.

THE MICROBIOME OF *L. PATELLA*

Sequencing of the *16S rRNA* gene and bulk metagenomic analysis have provided detailed information on the composition of a tremendous variety of microbial assemblages, ranging from the human body to marine environments. Similar studies performed on ascidian-associated microbial communities revealed a high bacterial diversity (Martínez-García et al., 2007; Donia et al., 2011a; Behrendt et al., 2012a), possibly due to distinct microenvironments as demonstrated in this study, and domination by selected phyla such as proteobacteria and cyanobacteria (Tait et al., 2007; Menezes et al., 2010; López-Legentil et al., 2011; Behrendt et al., 2012a). Combined molecular and microenvironmental measurements on *L. patella* demonstrated the coexistence of three very different microbial communities, separated by only a few millimeters of animal tissue (Behrendt et al., 2011): (i) a biofilm on the upper surface exposed to high irradiance, (ii) a cloacal cavity dominated by *Prochloron* spp. characterized by strong depletion of VIS and a dynamic chemical microenvironment, and (iii) a biofilm community covering the underside of the animal, where light is depleted of visible wavelengths and enriched in NIR. Further molecular analysis of the *L. patella* microbiome revealed bacteria with a rich genetic diversity, capable of producing significant amounts of secondary bioactive metabolites (Donia et al., 2011a) and earlier studies highlighted *Prochloron* as one of the key producers of such compounds (Schmidt et al., 2005). Further metagenomic investigations of *Prochloron* cells revealed an arsenal of functional gene cassettes coding for secondary metabolites across large geographical distances, with surprisingly little genomic diversion within the different *Prochloron* cells (Donia et al., 2011a). Even though *Prochloron* seems to functionally depend on its host, its genome contains a set of core genes similar to other cyanobacteria (Donia et al., 2011b), which, in all likelihood allows life outside of the ascidian-*Prochloron* association. Despite this, larger amounts of free-living *Prochloron* cells have only been reported once (Cox, 1986), while facultative relationships have been reported between non-ascidian invertebrates and *Prochloron* cells (Cheng and Lewin, 1984; Parry, 1986).

The abundance of *Prochloron* cells appears to underlie seasonal changes in temperature (McCourt et al., 1984) and more generally the distribution of cyanobacteria is found to be governed by the composition and availability of light (Stomp et al., 2007a,b). NIR-utilizing bacteria associated with ascidians are a prime example of such a highly specialized niche-partitioning: the Chl *d*-containing cyanobacterium *A. marina* was found to survive below didemnid ascidians in a light environment relatively enriched in NIR and depleted in VIS due to the overlying layer of Chl *a*/*b*-containing *Prochloron* cells (Kühl et al., 2005, 2007a; Behrendt et al., 2012a). Such microenvironmental patterns can even be extrapolated to larger biological frameworks: the sampling of *L. patella* associated biofilms along a depth gradient thus revealed a negative correlation between the abundance of NIR-utilizing phototrophs (*A. marina, Rhodospirillaceae*, *Rhodobacteraceae*, *Chloracidobacteria*) and water depth indicating that their abundance was strongly influenced by the availability of NIR, which is readily attenuated by seawater (Behrendt et al., 2012a). This highlights the need to perform ecologically relevant molecular surveys of microbial diversity on an appropriate (micro-) scale with accompanying metadata. In this context, microenvironmental data can inform both observations obtained by genomics and even the actual planning of such surveys. We have demonstrated that *Prochloron* lives in a niche experiencing strong fluctuations of the chemical microenvironment, generally experiencing hypoxia or anoxia inside their ascidian host under low light or dark conditions. Such microenvironmental settings thus point to a potential presence of a number of anaerobic processes and microbes.

In densely populated cyanobacterial communities experiencing periods of hypoxia/anoxia, N_2 fixation is often found (Stal and Zehr, 2008; Steunou et al., 2008). Diazotrophy has been reported several times in photosymbiotic ascidians, but whether *Prochloron* can fix N_2 remains unclear (Paerl, 1984; Koike et al., 1993; Kline and Lewin, 1999). Interestingly, genes associated with N_2 fixation were absent in the recently published *Prochloron* genome, apparently forcing it to rely on recycled nitrogen from its host via a number of nitrogen transformation pathways present in the genome (Donia et al., 2011b). This fits with the observation that ammonium is the major nitrogenous waste product of the ascidian host (Goodbody, 1974), which is also effectively taken up by *Prochloron* (Parry, 1985), while the presence of genes for urease and urea uptake point toward a potential role of urea in the nitrogen cycling between host and symbiont (Donia et al., 2011b). Diazotrophy may, however, occur in *Prochlorons* microenvironment as other N_2-fixing bacteria such as *Azospirillum brasilense* were found to reside in the cloacal cavity of *L. patella* (Behrendt et al., 2012a) and this may explain some of the earlier observations of N_2 fixation (Paerl, 1984). Nevertheless, *Prochloron* is

clearly the predominant inhabitant of the peribranchial space and inner cloacal cavities of *L. patella*, and we speculate that such diazotrophs may benefit from the strong O_2 dynamics imposed by *Prochloron*.

It remains to be investigated what type of dark metabolism *Prochloron* is thriving on during periods of low or no light and anoxia. Our data show that oxic dark respiration of *Prochloron* is limited due to strong O_2 depletion in its microenvironment. Under such conditions, cyanobacteria can rely on fermentation for their energy metabolism (Stal and Moezelaar, 1997). To our knowledge, the presence and activity of fermentative pathways in *Prochloron* have not been investigated. A first look into the *Prochloron* genome published by Donia et al. (2011b) actually revealed the potential presence of both mixed acid as well as lactate/pyruvate fermentation pathways (data not shown), which are known to occur in cyanobacteria (Stal and Moezelaar, 1997). Our finding of low pH in darkness support the speculation that fermentation may be an important part of *Prochloron* dark metabolism. Confirmation of this and further insight to the dark metabolism of *Prochloron* awaits more dedicated physiological studies of its carbon metabolism, preferentially in combination with further scrutiny of the *Prochloron* genome and subsequent transcriptome analyses. The same holds true for other aspects of *Prochloron* metabolism and biosynthesis, such as the synthesis of UV protective MAAs, abundant lipids, sterols, and bioactive secondary metabolites in *L. patella* (Donia et al., 2011a,b). While the presence of these pathways in *Prochloron* has been shown, we still know nothing about the diel dynamics of gene expression and enzyme synthesis in photosymbiotic ascidians and how particular microenvironmental controls play a regulatory role. A combination of microenvironmental analysis with genomics approaches (see, e.g., Steunou et al., 2008; Jensen et al., 2011) to further resolve the biology of *Prochloron* thus seems a very promising way to proceed.

Prochloron remains uncultivated despite numerous attempts since its discovery in 1975 (Hirose et al., 2009a). An hitherto unconfirmed report of successful cultivation argued that *Prochloron* lacked the ability to synthesize tryptophan (Patterson and Withers, 1982), while the recent *Prochloron* genome shows the presence of a full set of tryptophan anabolic genes (Donia et al., 2011b). Interestingly, Patterson and Withers (1982) also found cell division of *Prochloron* under acidic conditions with maximal growth at pH 5.5 under low irradiance of $\sim$110 µmol photons m^{-2} s^{-1} over a 18:6 h light-dark cycle. We found acidification of the *Prochloron* microhabitat in response to darkness, albeit only a few areas reached pH <6 (see, e.g., **Figures 7C,D**). In contrast, *Prochloron* photosynthesis is limited to a higher pH range with a maximum at pH 8–8.5 (Dionisio-Sese et al., 2001). Not much is known about the cell division cycle of *Prochloron*, besides one study reporting a diurnal rhythm with maxima of dividing cells in early morning and afternoon (Lewin et al., 1984), and we do not know how such cell division pattern is modulated by microenvironmental changes.

With the new knowledge on pH and O_2 dynamics presented here, it is possible to further optimize enrichment and cultivation scenarios better mimicking the natural habitat of *Prochloron* and to test new working hypotheses such as the importance of chemical and light gradients for *Prochloron* growth, and the importance of keeping *Prochloron* in a biofilm/aggregated growth mode for longer survival outside its host. A promising approach for enrichment and growth of *Prochloron* cells in gradients under controlled biofilm-like conditions could be based on immobilization of extracted *Prochloron* cells in alginate beads; an approach recently shown to work with *A. marina* (Behrendt et al., 2012b).

MATERIALS AND METHODS

FIELD SITE AND SAMPLE COLLECTIONS

Didemnid ascidians were sampled at low tide on the outer reef flat and crest off Heron Island (S23°26′0055, E151°55′0850), Great Barrier Reef in the Austral summer (January–February) during several field trips in the period 2001–2012. Specimen of *L. patella* were sample as larger intact specimens (5–20 cm^2; 5–20 mm thick) covering coral patches on the outer reef flat and down to $\sim$4 m depth on the reef crest (**Figure 1**). A more detailed description of sampling and field sites is given in Behrendt et al. (2012a).

Collection of didemnids was either done by hand or snorkeling. The samples were immediately transported in a bucket with sea water back to Heron Island Research Station, where they were transferred to outdoor aquaria continuously flushed with fresh aerated seawater (26–28°C) pumped in from the reef. Frequent exchange of water by flushing and continuous strong mixing of water in the aquarium is necessary to avoid degradation of the ascidians. To avoid high solar irradiance, the aquaria were covered by shading cloth dampening solar irradiance to $\sim$200–300 µmol photons m^{-2} s^{-1}. Under such conditions we could keep the sampled ascidians healthy and with actively photosynthesizing *Prochloron* for up to a week.

Measurements were done on small intact colonies of *L. patella* ($\sim$2–4 cm^2, 5–10 mm thick), as well as on specimens with an exposed fresh vertical cut through the test or, alternatively, on 1–2 mm thick vertical sections of *L. patella*. Transfer, slicing and cutting of specimen were done in a large beaker of seawater to buffer pH changes due to release of acid vacuoles. Relatively flat and homogeneous pieces of *L. pa*tella with a surface area of a few cm^2 were cut with a scalpel and immediately rinsed and submerged in filtered seawater. Cross-sections were cut from homogenous pieces with a razor blade for subsequent imaging. Generally, specimens showed a high maximal PSII quantum yield of >0.7 for several hours after such handling indicative of fast recovery and minimal stress on *Prochloron*.

ELECTRON MICROSCOPY

TEM

Specimens were fixed with 2% v/v glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2). Following isolation of suitable specimen blocks, the samples were rinsed three times in 0.15 M sodium cacodylate buffer (pH 7.2) and subsequently post-fixed in 1% w/v OsO_4 in 0.12 M sodium cacodylate buffer (pH 7.2) for 2 h. The specimens were dehydrated in graded series of ethanol, transferred to propylene oxide, and embedded in Epon according to standard procedures. Sections, $\sim$80 nm thick, were cut with a Reichert-Jung Ultracut E microtome and collected on

copper grids with Formvar supporting membranes. Ultra thin sections were collected on copper grids with Formvar supporting membranes and stained with uranyl acetate and lead citrate, and subsequently examined with a Philips CM 100 TEM (Philips, Eindhoven, Netherlands), operated at an accelerating voltage of 80 kV and equipped with an OSIS Veleta digital slow scan 2k × 2k CCD camera. Digital images were recorded with the ITEM software package.

FIB (focused ionbeam) SEM

Specimens were fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) and postfixed in 1% w/v OsO_4 with 1.5% potassium ferrocyanide. Following a rinse in dH_2O the specimens were stained en bloc in 1% Uranyl acetate over night, dehydrated in ethanol, and embedded in Epon according to standard protocols. The Epon blocs were mounted on aluminum stubs with colloidal carbon as an adhesive, sputter-coated with gold (Polaron SEM Coating Unit E5000), and imaged with a Quanta 3D FEG (FEI) operated at 5 kV using a vCD backscattered electron detector. Additionally, mounted cross-sections of the same samples were imaged on a Tecnai G2 20 Twin transmission electron microscope.

IMAGING OF VARIABLE CHLOROPHYLL FLUORESCENCE

Cross-sections through *L. patella* were placed in a small Petri-dish and covered by a thin (∼1–2 mm) layer of seawater and investigated with a variable chlorophyll fluorescence imaging system (I-PAM, Walz GmbH, Germany) consisting of a CCD camera, a LED ring light (with blue, red, and NIR LEDs), and a controlling unit connected to a PC running the dedicated imaging system software (Imaging-WIN 2.3, Walz GmbH, Germany). The blue LEDs (470 nm) provided both weak pulses of measuring light as well as defined levels of actinic light measured as downwelling photon irradiance, E_d, in the focus plane of the system with a calibrated irradiance meter (LI-250 and LI-192, LiCor, USA). A detailed description of variable chlorophyll fluorescence imaging systems is given elsewhere (Grunwald and Kühl, 2004; Ralph et al., 2005; Kühl and Polerecky, 2008; Trampe et al., 2011). The system enabled quantification of a proxy for PAR absorptivity, A, by measuring the reflected red light (R, 650 nm) and NIR (R_{NIR}, 780 nm) from the specimen/sample and calculating the ratio $A = 1 - (R/R_{NIR})$.

Based on the non-actinic imaging of the chlorophyll fluorescent yield before and during a strong saturation pulse, using weak modulated blue measuring light, several parameters characterizing the distribution and photosynthetic performance of oxyphototrophs in the ascidian specimens could be quantified and visualized. This included:

(1) The maximal quantum yield of PSII electron transport, $\Phi_{max} = (F_m\text{-}F_0)/F_m$, where F_0 is the minimal fluorescence yield and F_m is the maximal fluorescent yield of the dark-adapted sample measured prior to and during a strong saturation pulse, respectively;
(2) The effective quantum yield of PSII electron transport $\Phi_{PSII} = (F'_m - F)/F'_m$, where F is the fluorescent yield under a known level of blue actinic light and F'_m is the maximal fluorescent yield during a subsequent saturation pulse, respectively.

Based on these measurements, we calculated images of a range of derived photophysiological parameters characterizing photochemical and non-photochemical quenching of absorbed light energy in *Prochloron* and other oxyphototrophs present in the ascidians: a proxy for the relative photosynthetic activity was calculated from the relative PSII electron transport rate $rETR = \Phi_{PSII}\ E_d$ by taking the different absorptivity over the imaged specimen into account, as $PS = A\ \Phi_{PSII}\ E_d$.

The imaging system enabled acquisition of the mentioned parameters under a range of different actinic light levels applied to the samples over pre-defined time periods ranging from 10–20 s to 10 min. In this way, we could obtain information on both rapid and slow responses of photosynthetic performance. So-called rapid light curves (RLC) were obtained my measuring Φ_{PSII} at increasing irradiance using 10–20 s exposure to each irradiance level. Such light curves provide a snap-shot of the current photosynthetic capacity for handling light but should not be regarded similar to traditional photosynthesis vs. irradiance curves that are measured at steady state after longer incubation times (Schreiber et al., 1997). Such steady state light curves (SLC) of Φ_{PSII} vs. irradiance were obtained using 5–10 min long incubation times. Besides images, information averaged over particular regions of interest (ROI) was extracted with the system software (Image WIN 2.3, Walz GmbH, Germany).

MICROSCALE LIGHT MEASUREMENTS

Light propagation in the ascidian tissues was measured with fiber-optic scalar irradiance microprobes (Kühl, 2005) coupled to a fiber-optic spectrometer (QE65000, Ocean Optics, Dunedin, USA) for measurements of spectral scalar irradiance. Intact ascidian samples were fixed onto a black neoprene holder by thin insect preparation needles and placed in a flow chamber. The scalar irradiance microprobe was mounted in a manually operated micromanipulator (MM33, Märzhäuser, Wetzlar, Germany) and inserted into the ascidian tissue at an angle of 45° relative to the vertically incident light from a fiber-optic halogen lamp equipped with a collimating lens (KL2500, Schott, Germany). The solid test material of *L. patella* did not allow direct insertion of the spherical microprobe tip, and it was necessary to make a minute incision with the tip of a thin (29G) hypodermic needle prior to measurements.

All scalar irradiance measurements were normalized to the incident downwelling irradiance as measured with the scalar irradiance microprobe positioned in a black light well at a similar position in the light field as over the unic surface. Spectral measurement in different depths were also integrated over visible wavelengths (400–700 nm) driving oxygenic photosynthesis in *Prochloron* as well as over a region in the NIR (700–740 nm) that can be used by oxyphototrophs containing Chl *d* or *f*. This allowed calculation of depth profiles of VIS and NIR scalar irradiance in % of the incident downwelling irradiance in the respective spectral ranges.

MICROSCALE O_2 AND PH MEASUREMENTS

Microscale O_2 measurements were done with amperometric Clark-type O_2 microelectrodes (Revsbech, 1989) connected to a pA-meter (PA2000 or Microsensor Multimeter, Unisense,

Denmark). The O_2 microsensors exhibited a fast response time ($t_{90} < 0.5$ s), low stirring sensitivity (<1–2%) and had tip diameters of 25–50 μm. In *L. patella*, it was necessary to make an incision with a thin (29G) hypodermic needle prior to measurements. The O_2 microsensors were linearly calibrated from sensor readings in O_2-free solution (seawater amended with sodium dithionite) and in aerated seawater at experimental temperature and salinity (100% atmospheric saturation).

Microscale pH measurements were attempted with pH glass needle electrodes (pH-N, Unisense AS, Aarhus, Denmark; Kühl and Revsbech, 2001) connected to a high impedance mV meter (Keithley, USA) and calibrated at experimental temperature in standard pH buffers at pH 4, 7, and 10. The sensors exhibited a near-ideal Nernstian response of 55–59 mV per pH unit change. However, the pH sensors were too fragile for profiling pH in the sturdy tunic of *L. patella* and we observed apparent local release of acid vacuoles, when inserting the relatively large needle sensors, that changed the pH microenvironment dramatically down to pH 2–3 within seconds before slowly rising again to ambient pH over some minutes.

For microprofiling, the microsensors were mounted in a PC-controlled motorized micromanipulator system (Unisense, Denmark) that enabled automated profiling (in vertical steps of 50–100 μm) and data acquisition via dedicated software (Profix, Pyroscience, Germany).

IMAGING OF O_2 AND PH DISTRIBUTION AND DYNAMICS

The spatial distribution and dynamics of O_2 concentration and pH in *L. patella* was imaged on a vertical cut specimen, which was mounted tightly up against the transparent flow chamber wall with help of two hypodermic needles fixed in the soft chamber bottom. The planar optodes (see below) were positioned between the specimen and the inner wall of the flow chamber. The temperature in the flow chamber was kept constant at 26°C. Actinic light was provided vertically from above using a fiber-optic halogen lamp (LG-PS2, Olympus, Japan). To avoid interference, the lamp was briefly switched off during the image acquisition. The irradiance of the actinic light at the level of the ascidian test surface was determined in water with a scalar irradiance mini sensor (Model US-SQS/L, Walz GmbH, Effeltrich, Germany) coupled to a calibrated irradiance meter (ULM-500, Walz GmbH, Effeltrich, Germany). Photographic images of the vertically cut ascidian surface mounted against the transparent flow chamber were taken with a digital camera (Canon 5D, Canon, Japan).

Sensor materials

The fundamentals of sensor fabrication and solute imaging with planar optodes as well as details of sensor synthesis and physicochemical properties are described in detail elsewhere (e.g., Borisov et al., 2008, 2009a,b; Kühl and Polerecky, 2008; Kühl et al., 2007b, 2008; Mayr et al., 2009; Larsen et al., 2011; Staal et al., 2011; Fabricius-Dyg et al., 2012). Planar optodes for O_2 imaging were prepared by first dissolving 3 mg of platinum(II) tetra(4-fluoro)phenyltetrabenzoporphyrin (=PtTPTBPF; Borisov et al., 2008, 2009a) and 200 mg of polystyrene (MW 250,000, Aldrich, USA) in 1.8 g of chloroform. This "cocktail" was knife-coated onto a transparent poly(ethylene terephthalate) support foil (Mylar, Goodfellow, USA). The thickness of the sensing layer after evaporation of the solvent was ∼2.5 μm. The O_2 sensor chemistry possesses exceptional brightness due to high molar absorption coefficients and luminescence quantum yields of the indicators and enables excitation with red light (λ_{max} 615 nm). The NIR emission at ∼765 nm allows minimization of scattering effects and interfering chlorophyll fluorescence.

Planar optodes for pH imaging were prepared by first dissolving 3 mg of the coumarin dye Macrolex®Fluorescent Yellow (=MY; Simon and Werner GmbH)[1], 3 mg of lipophilic 1-hydroxypyrene-3,6,8-tris-bis(2-ethylhexyl)sulfonamide [=HPTS(DHA)$_3$; Borisov et al., 2009b] and 200 mg of Hydrogel D4 (Cardiotech)[2] in 1.8 g of ethanol:water (9:1 v/v). The pH sensor "cocktail" was also coated onto a transparent Mylar support foil. The thickness of the pH sensing layer after evaporation of the solvent was ∼7.5 μm.

Since the absorption spectrum of the basic form of the pH indicator perfectly matches the emission spectrum of the MY reference dye, Förster Resonance Energy Transfer (FRET) leads to the emission from HPTS(DHA)$_3$ at high pH (**Figure 9**). The protonated form of HPTS(DHA)$_3$ absorbs at about 450 nm, therefore, no FRET is observed at lower pH and the emission from MY is maximal. Thus, the system allows referenced ratiometric imaging of pH by monitoring the emissions from the two dyes – MY at ∼505 nm and HPTS(DHA)$_3$ at ∼555 nm.

Imaging of O_2

Planar O_2 optodes were read out with a lifetime imaging system [see details in Holst et al., 1998) employing a monochrome gated CCD camera (Sensicam-Sensimod, PCO, Kehlheim, Germany)] equipped with a Schneider-Kreuznach Xenoplan 1.4/23 CCTV-Lens and a R-720 long-pass filter (Edmunds Optics, USA) mounted in front of the lens. Two red-orange high power LEDs (617 nm, 1 W, Luxeon, Philips Lumileds, USA) were used for excitation, as controlled by a custom-built PC-controlled pulse-delay generator. A rapid lifetime determination method was used to determine the O_2-dependent luminescence decay time τ by measuring luminescence intensities in two time windows (1–41 and 26–66 μs, respectively) after the excitation pulse:

$$\tau = \frac{t_1 - t_2}{\ln\left(I_1 / I_2\right)}, \quad (1)$$

where t_1 and t_2 represent the time corresponding to the start of the first and the second window, respectively (1 and 26 μs in our setup), and I_1 and I_2 are the luminescence intensities acquired in the respective time windows.

A two point calibration (in air saturated and anoxic seawater, respectively) was used to calibrate the τ vs. O_2 oxygen response. A 2% w/w aqueous solution of sodium dithionite was used for deoxygenation. The following equation was used to obtain

[1] http://www.simon-und-werner.de
[2] http://www.cardiotech-inc.com

the calibration curve and to convert the measured decay times into pO_2:

$$\frac{\tau}{\tau_0} = \frac{f}{1 + K_{SV}\,[O_2]} + 1 - f, \quad (2)$$

where $f = 0.91$ (determined from the calibration for the PtTPTBPF/PS optodes obtained in the frequency domain measurements), τ_0 is the decay time in the anoxic solution (53.2 µs), and K_{SV} is the Stern–Volmer constant (0.0196 hPa^{-1}) as determined from the decay times at air saturation and under anoxic conditions under experimental temperature and salinity.

We used Matlab 7.10 (Mathworks Inc., USA) to calculate pseudocolor decay time images from the original data and, subsequently pseudocolor O_2 concentration images.

Imaging of pH

The pH optodes were read out with a new spectral camera system (Spectrocam, Ocean Thin Films, USA) consisting of a CCD camera combined with a fast-switching filter wheel (eight filter positions) and equipped with a Distagon TX2.8/25 mm ZF lens (Carl Zeiss AG, Germany). A long-pass glass filter (OG 490, Schneider-Kreuznach GmbH, Germany) was mounted in front of the lens to eliminate interference from the reflected excitation light. The pH optode was excited with blue light (λ_{max} 460 nm, as determined with a fiber-optic spectrometer, USB2000+, Ocean Optics, USA) from a torch (Bluestar, NightSea, Bedford, MA, USA). The emission of the pH sensor was monitored in two spectral windows: "window 1" (filter position with a Spectrocam 475/100 nm band width interference filter) and "window 2" (filter position with a Spectrocam 550/100 nm band width interference filter).

The emission of $HPTS(DHA)_3$ was imaged with the 550/100 nm filter. Notably, some emission from MY is also detected in this window (**Figure 9A**). The emission from MY was detected using the 475/100 nm bandpass filter. Thus, the effective window for monitoring the green fluorescence of MY was 490–525 nm. Subsequently, pH was evaluated by the ratio of the two windows: $R = (\text{window 2/window 1}) \times 1200$.

The pH optode was calibrated by ratiometric imaging in seawater at experimental temperature and salinity, wherein specific pH values were adjusted by addition of 0.1 M HCL or 1 M NaOH. The seawater pH at different calibration points was measured with a calibrated pH glass electrode connected to a pH meter (UB-10, Denver Instruments, USA). Calibration curves were obtained both for a fresh sensor foil and a sensor foil used in experiments to enable compensation for sensor drift due to dye bleaching. Image calculations and pseudocolor pH distribution images were done in *Matlab* 7.10 (Mathworks Inc., USA) by using individual calibration curves generated for each image. The pH of the seawater (8.03) was used for recalibration.

Calibration curves of sensor foils showed a shift in the intensity ratio after the experiment (**Figure 9B**), caused, e.g., by bleaching of the pH indicator during irradiation with actinic light. Therefore, the intensity ratio in the regions outside of the ascidian sample (seawater, pH 8.03) was used for recalibration. Thereby, an individual calibration curve was generated for each image and the drift was successfully compensated for. Note that the optimal dynamic range of the optodes is at pH 6–9. The sigmoidal calibration curve flattens at pH >9 and pH <6, and pH values obtained in these ranges are therefore prone to less accuracy.

Information on O_2 concentration and pH (average ± standard deviation) within particular ROI were extracted within *Matlab*.

AUTHOR CONTRIBUTIONS

Michael Kühl, Lars Behrendt, Sergey M. Borisov, Ulrich Schreiber, and Anthony W. D. Larkum designed and performed research; Michael Kühl, Erik Trampe, Klaus Qvortrup, Ulrich Schreiber, Sergey M. Borisov, and Ingo Klimant contributed new reagents/analytic tools; Michael Kühl, and Sergey M. Borisov analyzed data; and Michael Kühl, Lars Behrendt, Sergey M. Borisov, and Anthony W. D. Larkum wrote the paper with editorial help from all co-authors.

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Light gradients and optical microniches in coral tissues

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Light quantity and quality are among the most important factors determining the physiology and stress response of zooxanthellate corals. Yet, almost nothing is known about the light field that *Symbiodinium* experiences within their coral host, and the basic optical properties of coral tissue are unknown. We used scalar irradiance microprobes to characterize vertical and lateral light gradients within and across tissues of several coral species. Our results revealed the presence of steep light gradients with photosynthetically available radiation decreasing by about one order of magnitude from the tissue surface to the coral skeleton. Surface scalar irradiance was consistently higher over polyp tissue than over coenosarc tissue in faviid corals. Coral bleaching increased surface scalar irradiance by ~150% (between 500 and 700 nm) relative to a healthy coral. Photosynthesis peaked around 300 μm within the tissue, which corresponded to a zone exhibiting strongest depletion of scalar irradiance. Deeper coral tissue layers, e.g., ~1000 μm into aboral polyp tissues, harbor optical microniches, where only ~10% of the incident irradiance remains. We conclude that the optical microenvironment of corals exhibits strong lateral and vertical gradients of scalar irradiance, which are affected by both tissue and skeleton optical properties. Our results imply that zooxanthellae populations inhabit a strongly heterogeneous light environment and highlight the presence of different optical microniches in corals; an important finding for understanding the photobiology, stress response, as well as the phenotypic and genotypic plasticity of coral symbionts.

Keywords: coral photobiology, bio-optics, microenvironment, tissue optics, zooxanthellae, microsensor, microgradients, ecophysiology

INTRODUCTION

Coral reefs are among the most productive and diverse ecosystems on Earth and their evolutionary success can be largely attributed to the successful interaction between scleractinian corals and their associated microorganisms, most importantly their microalgal photosymbionts (zooxanthellae) belonging to the dinoflagellate genus *Symbiodinium*. The quantity of light is a key environmental parameter regulating the nature of this photosymbiosis (Falkowski et al., 1990). Under optimal irradiance regimes, light stimulates symbiont photosynthesis, which provides organic carbon for the coral animal that in turn provides metabolic waste products supporting zooxanthellae photosynthesis (Muscatine et al., 1981). Excess quantities of light, however, readily lead to photoinhibition and can damage the photosynthetic apparatus. Light in combination with elevated temperature can lead to the expulsion of the zooxanthellae (and/or pigment degradation) and the breakdown of the symbiosis (Lesser, 1996; Jones et al., 1998; Warner et al., 1999). This breakdown, termed coral bleaching, has been intensively studied over the last decades, including a primary focus on the photobiology of zooxanthellae (Glynn, 1996; Brown, 1997; Hoegh-Guldberg, 1999). Despite such efforts, it is surprising that virtually nothing is known about the actual light regime surrounding the zooxanthellae *in hospite*, i.e., within the coral tissue, albeit the light microenvironment is a central control factor of the photo- and stress physiology of zooxanthellae and their coral hosts.

The optical environment within the host tissue is likely to vary substantially in relation to the ambient macro-environment. First, direct micro-scale measurements of photon scalar irradiance (i.e., the integral quantum flux incident from all directions about a given point) on the coral tissue surface revealed scalar irradiance values reaching up to 200% of the incident downwelling photon irradiance (Kühl et al., 1995). Such enhancement is currently thought to mainly result from multiple scattering of photons in the coral skeleton below the tissue (Enriquez et al., 2005). The aragonite skeleton scatters light isotropically so that photons interacting with the skeleton are diffusely backscattered into the tissue (Enriquez et al., 2005). Diffuse scattering increases the path length of photons per vertical distance traversed, i.e., it enhances the average residence time of photons at a given depth horizon and can thereby lead to local enhancement of scalar irradiance (Kühl and Jørgensen, 1994; Enriquez et al., 2005). It is currently assumed that the light field within the coral tissue is diffuse and uniformly enhanced over the incident irradiance (Enriquez et al., 2005; Teran et al., 2010). However, the optical environment within the coral may be more complex as tissue–light interactions and the optical properties of coral tissue remain largely unexplored.

Photons interacting with tissue can have three different fates: (i) simple unimpeded transmission; (ii) absorption followed by either red-shifted re-emission (as fluorescence or phosphorescence), heat dissipation or dissipation via photochemical reactions

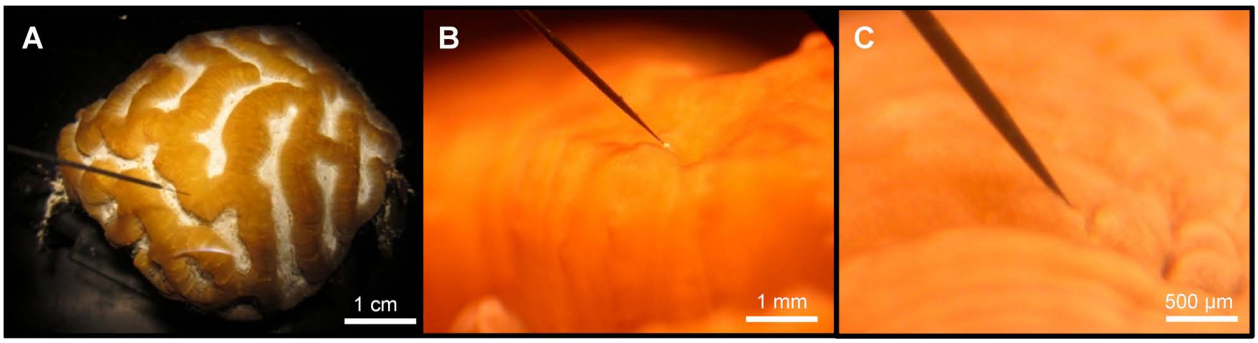

FIGURE 1 | Micro-scale scalar irradiance measurements on corals. **(A)** Overview showing a small fragment of *Platygyra lamellina* with the scalar irradiance microprobe positioned at the coral tissue surface at a 45° angle, **(B)** the spherical microsensor tip (white bulb; 100 µm) at the surface of coenosarc tissue, and **(C)** the microprobe inserted into coral tissue.

such as photosynthesis or radical formation; (iii) scattering and diffraction leading to a redirection of photons out of their original path. The occurrence of these events is determined by a complex interplay between the nature and direction of incident light and the optical properties of the given tissue (Wilson and Jacques, 1990). The optical properties of living tissue are best studied for human skin, but also well-described for terrestrial plants (Anderson and Parrish, 1981; Vogelmann, 1993) as well as aquatic sediments and biofilms (Kühl and Jørgensen, 1994). The development and use of fiber-optic microprobes (Vogelmann et al., 1991; Kühl, 2005) has facilitated experimental investigation of light microenvironments and optical properties within such systems (Vogelmann and Björn, 1984; Vogelmann, 1993; Vogelmann et al., 1996). Besides a few preliminary measurements (Kühl et al., 1995; Kaniewska et al., 2011), comparable studies on coral tissue are lacking. Kaniewska et al. (2011) mainly focused on comparing larger scale heterogeneity of light fields in different corals and presented only few spot measurements of scalar irradiance at a fixed depth in the coral tissue and no detailed vertical or lateral profiling was done. The presence and nature of micro-scale heterogeneity in coral light fields, both laterally over different coral tissue types and vertically within a given tissue type, have thus not been resolved.

Here we used scalar irradiance microprobes (Vogelmann and Björn, 1984; Lassen et al., 1992) to characterize the spectral light field and light penetration in coral tissues. The specific aims were (1) to directly measure light penetration in tissue of corals belonging to the family Faviidae, (2) investigate the effect of tissue type (coenosarc and polyp tissue) and loss of pigmentation (bleaching) on light microenvironments for a variety of abundant coral species, and (3) investigate how gradients of light and photosynthesis within coral tissue align with each other. Our results provide the first insight into the basic optical properties of coral tissue and describe the *in hospite* optical microenvironment of corals from a zooxanthellar perspective.

RESULTS

LIGHT FIELDS SURROUNDING CORAL TISSUE

Spectral scalar irradiance at the coral tissue surface (**Figure 1**) differed markedly between tissue types (i.e., coenosarc and polyp) both within a coral species and between species, despite identical regimes of incident collimated irradiance (**Figure 2**). Generally, there was an enhancement in scalar irradiance over incident irradiance reaching maximum values of ~180% for photosynthetically active radiation (PAR, 400–700 nm) and ~250% for near-infrared radiation (NIR, 700–800 nm). Scalar irradiance levels in faviid corals were systematically higher over polyp tissue than over coenosarc tissue for both PAR and NIR (**Figures 2 and 3A,B**). For example, the scalar irradiance measured over polyp tissue of *Favites abdita*, was about 1.4 and 1.3 times higher than over coenosarc tissue for PAR and NIR, respectively (**Figures 3A,B**). In contrast, no differences between polyp and coenosarc tissue were present in *Pocillopora damicornis* (**Figures 3A,B**). Characteristic absorption peaks of Chl *a* (430–440, 675 nm), Chl *c* (460 nm), and the carotenoid peridinin (480–490 nm) were found at the surface of faviid corals; these peaks were especially pronounced over polyp

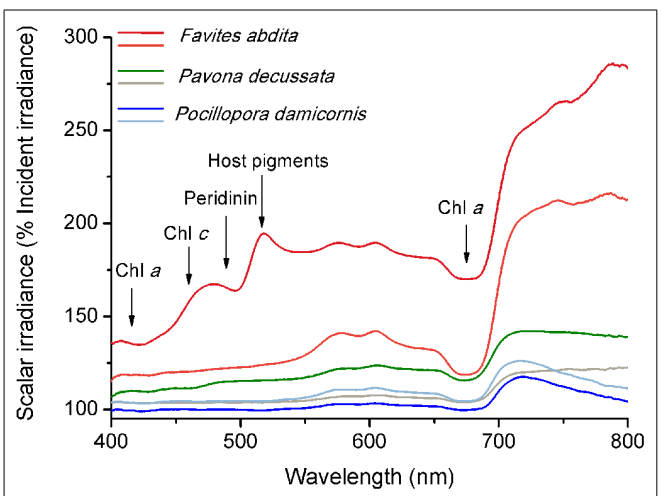

FIGURE 2 | Micro-scale spectral scalar irradiance at the tissue surface of different corals. Data were normalized to the incident downwelling spectral irradiance, E_d. Note that scale begins at 100% E_d. Dark and light color tones represent measurements made on polyp and coenosarc tissue, respectively. Arrows show major absorption wavelengths of peridinin (480–490 nm), chlorophyll *c* (460 nm) and chlorophyll *a* (435–440, 675 nm), and emission/reflectance of host pigments (480–590 nm).

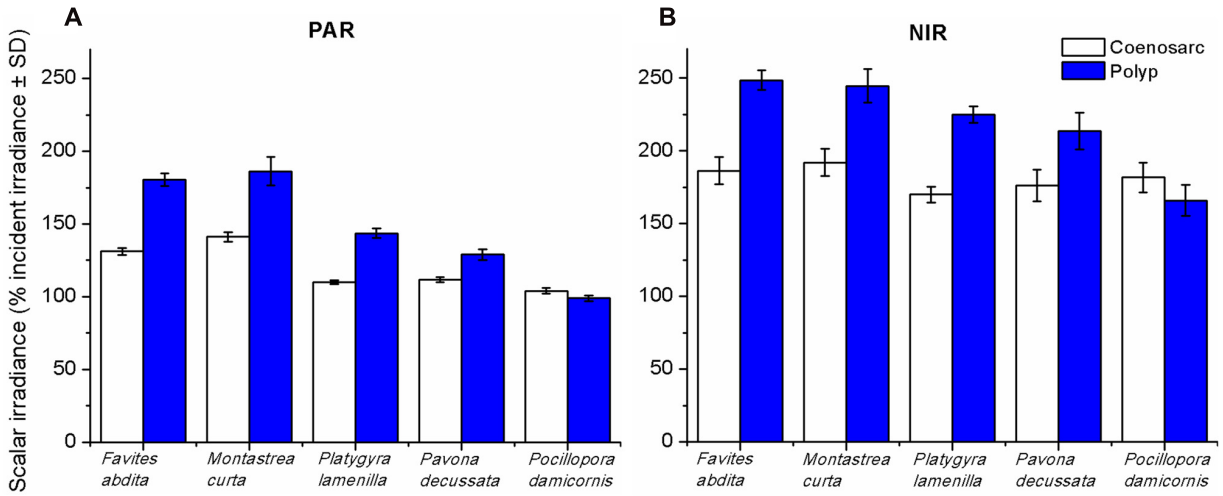

FIGURE 3 | Integrated scalar irradiance (in % of downwelling irradiance) at the tissue surface of corals. (A) PAR (photosynthetically available radiation, 400–700 nm) and **(B)** NIR (near-infrared radiation, 700–800 nm). Data are means ± SD ($n = 9$). Measurements were done at the surface of the coenosarc (white bars) and polyp (blue bars) tissue, respectively.

tissue. In comparison, spectra of *Pavona decussata* and *Pocillopora damicornis* showed less distinct spectral signatures (**Figure 2**). More detailed horizontal mapping of scalar irradiance across the tissue surface (from polyp mouth over walls to coenosarc) revealed strong small-scale heterogeneity (**Figure 4**), but consistent results were obtained between the two different tissue types (coenosarc and polyp mouth tissue). Pigmentation across the polyp walls appeared heterogeneous (patchy distribution of host pigments) as were the measurements of scalar irradiance. When the sensor was placed within a tissue fold (i.e., sphere entirely covered by tissue), irradiance was significantly attenuated (**Figure 4**).

EFFECTS OF CORAL BLEACHING ON TISSUE SURFACE SPECTRAL SCALAR IRRADIANCE

Loss of pigmentation (bleaching) led to a further increase in the tissue surface scalar irradiance of corals reaching ∼150% of the scalar irradiance levels of a healthy coral at wavelengths between ∼500 and 700 nm (**Figure 5**). However, in the blue region of visible light (∼400–500 nm) and in the NIR, bleaching only led to a slight (10–20%) increase in scalar irradiance. Bleaching affected the light field of coenosarc tissue to a greater extent than that of polyp tissue with greatest deviations of >10% occurring between ∼470–510 and 720–800 nm.

SPECTRAL SCALAR IRRADIANCE WITHIN CORAL TISSUE

Our microprofiles revealed the presence of strong light gradients within coral tissue (**Figures 6A,B**). Over the visible range (PAR, 400–700 nm), scalar irradiance was attenuated toward the skeleton. For coenosarc tissue, scalar irradiance of PAR decreased from ∼132 to 74% of the incident downwelling irradiance over a distance of 400 μm. Such decrease in PAR was even more pronounced for a 1100-μm thick polyp tissue, where PAR

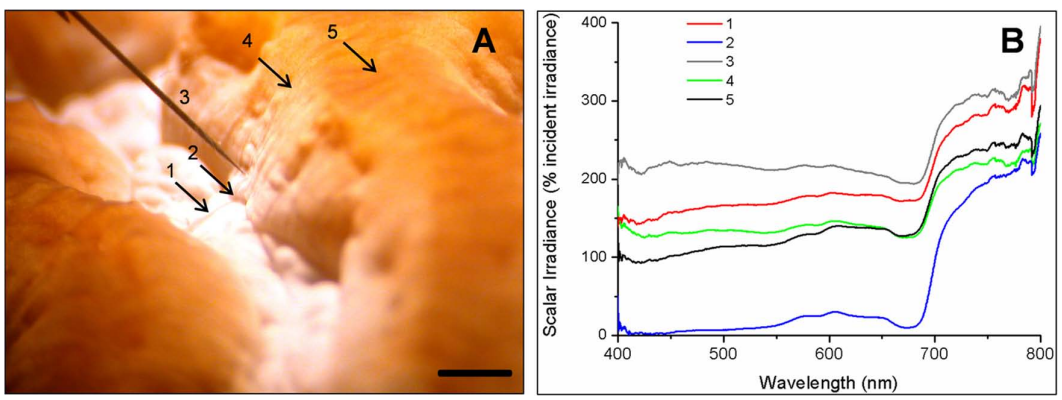

FIGURE 4 | Mapping of spectral scalar irradiance heterogeneity along one coral polyp in *Platygyra lamellina*. (A) Photograph detailing the five different measurement positions along a horizontal tissue surface gradient (indicated as arrow or sensor tip, 1–5). Black scale bar is 2 mm. **(B)** Single scalar irradiance spectra (400–800 nm) at the measurement points (1–5).

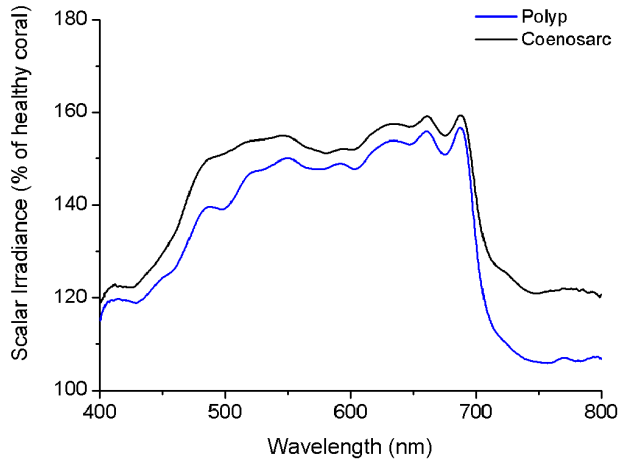

FIGURE 5 | Effect of bleaching on the tissue surface scalar irradiance of a coral. The figure shows spectral scalar irradiance for coenosarc (black) and polyp (blue) tissue of a bleached *Goniastrea aspera*, expressed as percentage of healthy coral.

scalar irradiance decreased from ∼176 to 16% of the incident downwelling irradiance. In many cases, we noted a subsurface maximum ∼50–100 μm below the tissue surface, where no attenuation or even an increase in scalar irradiance occurred. This subsurface maximum was most pronounced at wavelengths around 550–650 nm (**Figure 6A**), i.e., outside major absorption peaks of photopigments. Most of the light was absorbed in the upper tissue layers and spectra close to the skeleton were more similar in intensity (**Figures 6A,B**). In the NIR region, no attenuation occurred within coenosarc tissue and high values of around 220–240% of incident downwelling irradiance remained (**Figures 6A and 7A**). In contrast, NIR did significantly attenuate from surface to skeleton inside polyp tissue from ∼306 to 124% of the incident downwelling irradiance (**Figure 6B**).

OXYGEN MICROENVIRONMENT AND PHOTOSYNTHESIS WITHIN CORAL TISSUE

Oxygen measurements within illuminated coral tissue revealed a steady increase in O_2 concentration from the tissue surface toward the skeleton (**Figure 7B**). Maximum O_2 concentrations at the tissue–skeleton interface reached ∼800 μM (∼400% air saturation), which was about 240 μM higher than the O_2 concentration at the tissue surface. Gross photosynthesis increased with distance from the surface until ∼300 μm within the tissue, where it peaked at ∼18.9 (±4.7 SE) nmol O_2 cm^{-3} s^{-1}. The peak in gross photosynthesis correlated with a zone in the tissue exhibiting the highest scalar irradiance attenuation (compare **Figures 7A,B**). Photosynthesis was lowest at the coral tissue surface and at the tissue–skeleton interface (8.3 ± 2.5 and 10.9 ± 3.0 nmol O_2 cm^{-3} s^{-1}, respectively; **Figure 7B**).

The relationship between incident downwelling photon irradiance and the photon scalar irradiance for visible wavelengths (400–700 nm) was measured at the tissue–skeleton interface ∼1000 μm within polyp tissue. There was a constant linear relationship between incident irradiance and the scalar irradiance level at the tissue–skeleton interface ($r^2 > 0.99$; **Figure 8**). Over the range of experimental downwelling photon irradiances (150–2000 μmol photons m^{-2} s^{-1}) only about 1/10 was measured as photon scalar irradiance within this optical niche.

DISCUSSION

In this study, we used fiber-optic microprobes to obtain the first detailed measurements of vertical and lateral light gradients within and across coral tissues in several species. While the chemical

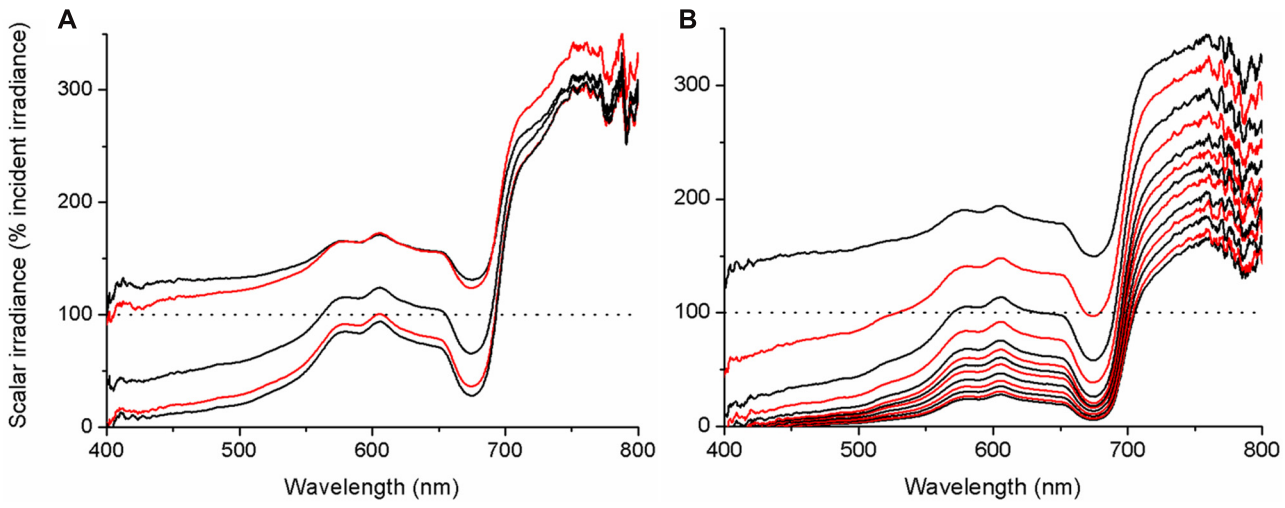

FIGURE 6 | Microprofiles of spectral scalar irradiance within coral tissue. Representative profiles measured in the coral *Montastrea curta* within **(A)** coenosarc (tissue thickness ∼400 μm) and **(B)** polyp tissue (tissue thickness ∼1200 μm). Scalar irradiance was normalized to the incident downwelling spectral irradiance, E_d; dotted line represents 100% E_d. The uppermost spectrum (black) represents measurements taken at the coral surface and subsequent spectra correspond to increments of 100 μm with the lowermost spectrum equaling measurements over the coral skeleton. Spectra are colored in an alternating fashion (black–red) for clarity.

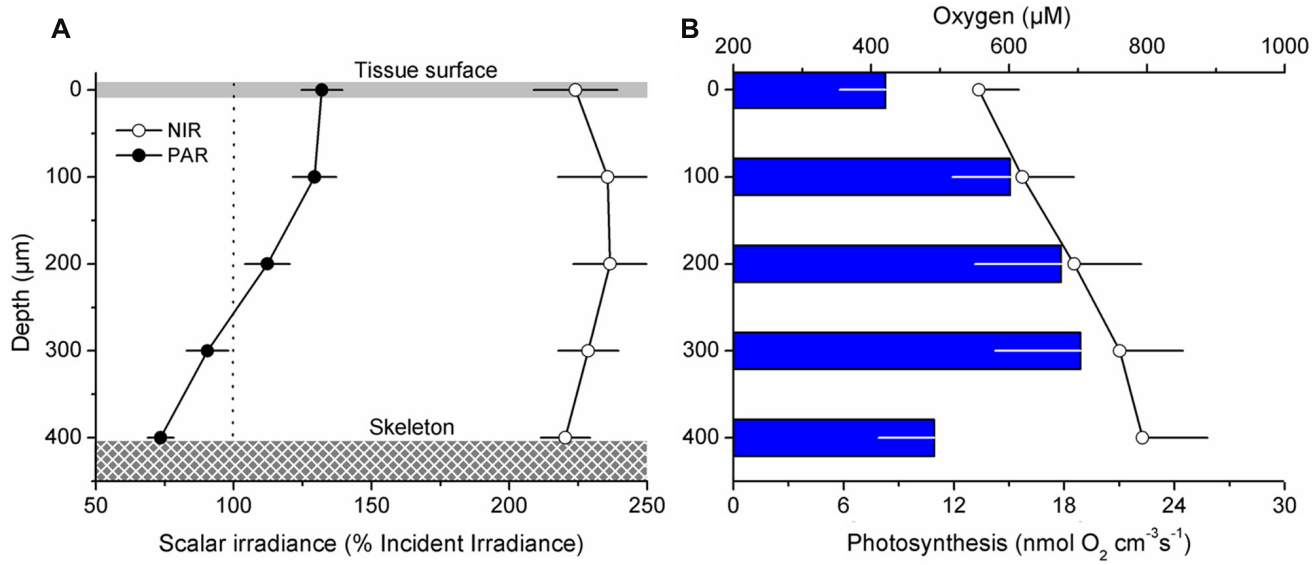

FIGURE 7 | Microgradients of light, O_2, and photosynthesis within coral tissue. Microprofiles were measured within ~400 µm thick coenosarc tissues of the coral *Montastrea curta* from just above the skeleton (depth 400 µm) to the coral surface (depth 0 µm). Data points are mean values (±SE; $n = 4$) **(A)** Mean photosynthetically available radiation (PAR, 400–700 nm; closed symbols) and near-infrared radiation (NIR, 700–800 nm; open symbols) scalar irradiance profiles. Scalar irradiance was normalized to the incident downwelling irradiance. **(B)** Average O_2 concentration (µM; open symbols) and gross photosynthesis (nmol O_2 cm^{-3} s^{-1}; blue bars). For clarity error bars of O_2 concentration and photosynthesis are only + and −, respectively. The downwelling photon irradiance E_d (PAR) was 640 µmol photons m^{-2} s^{-1}.

microenvironment of corals has been explored in several studies since microsensors were introduced to coral research (Kühl et al., 1995), only a few examples of scalar irradiance measurements in corals have been published and these have been hampered by difficulties in entering and/or precise positioning in the tissue (Kühl et al., 1995; Kaniewska et al., 2011). Hitherto, tissue effects on coral light fields have largely been ignored in coral optics studies that have mostly focused on the role of diffuse backscatter from the coral skeleton and from the coral tissue into the surrounding seawater (Enriquez et al., 2005; Teran et al., 2010). Combining micro-incision with scalar irradiance profiling, we have now unequivocally demonstrated the presence of light gradients in corals and present the first evidence that tissue optics is an important factor to consider in coral photobiology.

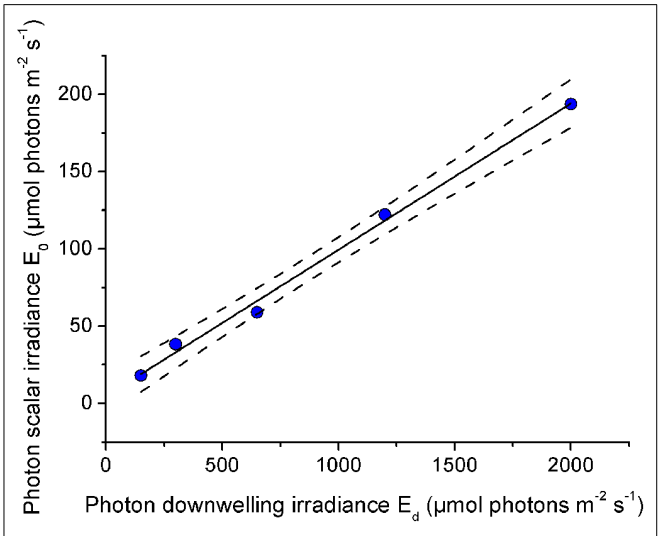

FIGURE 8 | Effect of incident photon irradiance on within tissue photon scalar irradiance (400–700 nm). The microsensor was positioned at a depth of 1000 µm within the polyp tissue just above the skeleton of the coral *Platygyra lamellina*. Black line illustrates a linear fit ($r^2 > 0.99$) and dashed lines represent ±95% confidence intervals.

Direct *in hospite* micro-scale light measurements in corals differ from predictions in previous modeling studies, which have calculated that internal irradiance is homogenously enhanced compared to the external environment based on known downwelling irradiance regimes (Enriquez et al., 2005; Teran et al., 2010). We show that a clear spatial stratification exists within coral tissue, where scalar irradiance in the upper coral tissue layers (0–100 µm) can reach up to 200% of incident downwelling irradiance, whilst lower cell layers are subject to more light-limiting conditions (**Figures 6B and 8**). Our results thus suggest that the light microhabitat of corals is not only determined by the properties of the skeleton, but also largely by the characteristics of the tissue.

Until now, coral tissue has simply been treated as a thin layer of light absorbing particles (i.e., *zooxanthellae*) on top of the light-diffusing skeleton and overlain by seawater (Teran et al., 2010). We know from other systems, however, such as plant leafs or animal skin, that the properties of the tissue itself can significantly enhance light fields at the tissue interface due to scattering and internal reflection (Anderson and Parrish, 1981; Vogelmann et al., 1996; Spilling et al., 2010). The peak of scalar irradiance observed here in the upper cell layers suggests that substantial scattering

and photon-trapping must occur at the tissue–water interface, potentially resulting from a mismatch in the refractive index of coral tissue and water (Kühl and Jørgensen, 1994). Nevertheless, our results also confirm that the earlier-reported diffuse scattering component of the skeleton is functional *in hospite* (Enriquez et al., 2005), as seen by a decrease in light attenuation toward the skeleton surface (**Figure 6B**). The occurrence and significance of skeleton backscatter is further exemplified by the continuous enhancement of NIR throughout the coenosarc tissue, where no pigments are present that absorb over these wavelengths (**Figure 7A**).

We hypothesized that with increasing incident irradiance, within-tissue PAR would increase exponentially as more light would be transmitted through the tissue and interact with the skeleton, thereby increasing the relative importance of backscattered light from the skeleton at the tissue–skeleton interface. However, we found a constant linear relationship between PAR at the tissue–skeleton interface and incident PAR (**Figure 8**). Photons are thus efficiently absorbed before they get scattered by the skeleton, indicating that the coral tissue itself also contributes to the high efficiency of light absorption found in corals (Stambler and Dubinsky, 2005). Scalar irradiance at the tissue surface increased by 150% in a bleached coral relative to the surface scalar irradiance in a healthy coral (**Figure 5**). This was less than expected according to coral skeleton scattering theory (Teran et al., 2010) and again suggests that other light redistributing mechanisms occur within the tissue. However, the nature of light gradients and thus the relative importance of tissue vs skeleton optics will be variable. Coral tissue varies in thickness, metabolite composition, symbiont and host pigment distribution, and abundance, all of which modulate coral tissue optics. Additionally, the role of skeleton optics is variable due to differences in morphology and density. For instance, thick corallite walls guide more light into the coral interior, whilst more dense structures facilitate diffuse backscattering (Highsmith, 1981). Therefore, the optical microenvironment within corals is the result of a complex interplay between skeleton and tissue optical properties, which clearly deserves further investigations.

Coral tissue surface scalar irradiance differed on a spatial scale between coral species and tissue types, despite identical incident irradiance regimes (**Figures 3A,B**). Since we excluded the potential for any interference with colony and/or macro-scale light-regulating factors such as colony morphology and orientation (Anthony et al., 2005), we conclude that the observed differences are caused by micro-scale optical properties of coral tissue and skeleton. In faviid corals, host pigments are often locally concentrated toward the polyp mouth (e.g., Salih et al., 2000; Oswald et al., 2007; but see spectral signatures in **Figure 2**). The enhanced tissue surface scalar irradiance of polyp over coenosarc tissue may be explained partly by the presence of such pigments, which effectively reflect, fluoresce, and scatter light (Schlichter et al., 1988; Salih et al., 2000).

Previous studies have shown the presence of tissue type-related spatial heterogeneity in photosynthesis (Ralph et al., 2002; Hill et al., 2004; Al-Horani et al., 2005). For instance, in the coral *Galaxea fascicularis* O_2 production was shown to be about 10 times higher over polyp than over coenosarc tissue (Al-Horani et al., 2005). Such differences may likely be related to distinct light microenvironments in the coral tissue. For productivity comparisons between species under identical incident irradiance regimes it appears crucial to consider the ability of corals to modulate their own light regime by skeleton structure and tissue organization/movement (**Figures 3A,B**). Tissue and skeleton optical properties have a strong effect on the local light environment that may partly explain observed species- and tissue type-related differences in photosynthesis.

Our results show that *Symbiodinium* populations, inhabiting oral and aboral coral tissue layers of faviid corals, experience steep light gradients with scalar irradiance reaching down to 10% of the surface irradiance in deeper tissue layers (**Figure 6B**); the vertical attenuation of light observed in the coral tissue over a few hundred microns is comparable to the reduction in irradiance that occurs between surface waters and >25 m depth in oceanic waters (Kirk, 1994). Our findings thus call for a revision of the current view on the optical environment surrounding zooxanthellae.

On the scale of a single colony, irradiance gradients between light exposed and shaded tissue can lead to both a distinct distribution of *Symbiodinium* clades and/or differential photoacclimation of the latter (Rowan et al., 1997; Toller et al., 2001; Ulstrup et al., 2006; Sampayo et al., 2007). The potential for such mechanisms occurring within tissue on a vertical micro-scale, for instance between oral and aboral tissue layers, is not known for corals, but is well-studied for terrestrial leaves (Schreiber et al., 1996). It is, e.g., known that shade-adapted chloroplasts exist in the lower tissue layers of sun-adapted leaves and chloroplasts deep within leaves are photoacclimated to local irradiance regimes (Terashima, 1989). We found that maximum rates of photosynthesis occurred in lower parts of coral tissue and not at the surface where scalar irradiance was at its maximum (**Figure 7B**). In fact, the spatial relationship between photosynthesis and light observed here is similar to results obtained from spinach leaves where photosynthetic O_2 production showed a peak deep within the leaf, whilst irradiance maxima were obtained at the top part of the leaf (Nishio et al., 1993). These findings underscore the potential for photoacclimation to different light microclimates within coral tissue.

Clades and sub-clades of *Symbiodinium* exhibit a range of light-harvesting strategies (Reynolds et al., 2008; Ragni et al., 2010; Kraemer et al., 2012) and it will be interesting in the future to ascertain the location of various clades in coral species that harbor more than one clade, relative to the actual light field characteristics. The optical environment is a primary factor controlling the activity and distribution of phototrophic organisms and the presence of intratissue light gradients must have an effect on the ecophysiology of zooxanthellae in yet unknown ways.

Our results also have implications for the understanding of coral bleaching patterns. It has been observed that thick-tissued corals survive stress events better than thin-tissued ones (Loya et al., 2001). It has also been hypothesized that thick coral tissue could provide sheltered light environments for resident zooxanthellae, thereby increasing stress resilience and the survival of thick-tissued corals (Hoegh-Guldberg, 1999). We show here that thick-tissued corals do indeed harbor such sheltered optical microniches (**Figures 6A,B**). This photoprotection is substantial as even under conditions of stressful excess radiation (incident PAR irradiance levels of ∼2000 μmol photons m^{-2} s^{-1}) thick coral

tissue can harbor low light niches for photosynthesis experiencing about 1/10 of incident irradiance (200 μmol photons m^{-2} s^{-1}; **Figure 8**).

Yet another option is that habitat heterogeneity is favored in thick-tissued corals, which in turn leads to a larger symbiont pool with diverse phenotypic or genotypic characteristics and stress resilience (Rowan et al., 1997). We found habitat heterogeneity both in the optical and the chemical environment (**Figures 7A,B**). The coral skeleton represents a diffusion barrier for chemical species, which will lead to a relative build-up of gases in lower tissue layers toward the tissue–skeleton interface as shown here by an increasing O_2 concentration up to ∼400% air saturation (**Figure 7B**). Thus, tissue thickness will favor microenvironmental heterogeneity. Whether this then favors a greater pool of symbiont populations (or subpopulations) and if this translates to increased stress resilience remains to be investigated.

In conclusion, we show here the first evidence for the presence of strong light gradients within the tissue of symbiotic corals. The optical properties of coral tissue have an important role in controlling microenvironmental light fields within corals. Our results imply that zooxanthellae within one single polyp can be subject to different light microenvironments with irradiance levels spanning over one order of magnitude. These results call for a revision of our current understanding of the interaction between light and corals and provide the very basis for future investigations on microenvironmental optical controls of coral photo- and stress physiology.

MATERIALS AND METHODS

CORAL SAMPLES

Corals were collected from shallow waters (<3 m depths) on the reef flat of the Heron Island lagoon, Great Barrier Reef, Australia (152°06′E, 20°29′S). We selected several species of faviid corals (*Favites abdita*, *Goniastrea aspera*, *Montastrea curta*, *Platygyra lamellina*) suitable for intratissue microsensor measurements (i.e., thick tissue and minimal mucus secretion; Alieva et al., 2008). We also sampled small coral fragments (<5 cm diameter) of the branching *Pocillopora damicornis* (Pocilloporidae) and the foliaceous *Pavona decussata* (Agariciidae) because they have contrasting micro-scale properties (pigment composition and microtopography) compared to faviids. Samples were transported to the permanent coral holding facility at the University of Technology, Sydney, where corals were maintained under continuous flow at 25°C, salinity of 33 and a photon irradiance of 200 μmol photons m^{-2} s^{-1} (400–700 nm; 12/12 h light–dark cycle).

SCALAR IRRADIANCE MICROSENSOR MEASUREMENTS

Experiments were conducted with coral fragments placed in a custom-made black acrylic flow chamber supplied with seawater (as above) at a flow velocity of ∼3 cm s^{-1}. Samples were illuminated vertically from above with a collimated light beam from a fiber-optic tungsten-halogen lamp (KL-2500, Schott GmbH, Germany), equipped with a heat filter and a collimating lens. The complete set-up was covered with black cloth to avoid stray light. Fiber-optic scalar irradiance microprobes with a spherical tip diameter of 80–100 μm were used to map light microenvironments in the corals (Lassen et al., 1992). The microprobes were mounted on a PC-controlled motorized micromanipulator for automatic profiling (Pyro-Science GmbH, Germany), at an angle of 45° relative to the vertically incident light (to avoid self-shading, see Lassen et al., 1992). The micromanipulator was fixed onto a heavy-duty vibration-free metal stand. Scalar irradiance spectra were recorded with the microprobes connected to a PC-controlled fiber-optic spectrometer controlled by the manufacturers software (USB2000+ and *Spectrasuite*, Ocean Optics, USA). Positioning of the microprobe was facilitated by the manufacturers software (Profix, Pyro-Science GmbH, Germany).

Scalar irradiance at the coral tissue surface was mapped at coenosarc (tissue connecting two polyps) and polyp tissue for the investigated coral species (**Figure 1**). These tissue types were chosen because of known differences in photobiology and pigment composition (e.g., Hill et al., 2004; Ulstrup et al., 2006). First, reference measurements of the incident downwelling irradiance (E_d) were done over a black non-reflective surface as described previously (e.g., Kühl and Jørgensen, 1992), followed by measurements at the coral surface. The coral surface was defined as the depth where the sphere of the sensor just touched the tissue. By rearranging the aquarium and/or the coral, but not the sensor or the incident light, we were able to measure coral surface irradiance at the identical spot in the incident light field, where E_d was measured, thereby avoiding potential artifacts due to heterogeneities in the incident light field. Differences in scalar irradiance were thus solely related to the optical properties of corals. Since we were interested in understanding how coral micro-scale properties and not gross morphology (i.e., growth form, etc.) modulate tissue light regimes, we only measured in locations that were directly exposed to the vertically incident collimated light beam. For each of the measured coral species, nine replicate measurements were done on randomly chosen coral polyps.

To investigate the impact of reduced pigmentation on coral tissue optics, we also measured a bleached and normally (dark-brownish) pigmented piece of *Goniastrea aspera*. The bleached coral originated from an experimental coral tank and was assessed to be severely bleached according to the color reference chart (score D1 = Chl *a* density: <1 μg cm^{-2}, cells: <1 × 10^5 cm^{-2}; see Siebeck et al., 2006). The tissue structure was visually assessed as healthy and uncompromised.

For measuring light gradients inside the coral tissue, a microincision had to be made in order to allow smooth tissue penetration by the sensor without indentation – a major problem in earlier attempts to measure light profiles in coral tissue. We found that a double-edged diamond knife (60°, blade thickness 200 μm, effective blade thickness <100 μm; ProSciTech Pty Ltd, USA) was very suitable for such purpose. Diamond knifes are commonly used in tissue micro-surgery because they perform highly precise and minimally invasive cuts. Incisions were made carefully under a dissecting microscope with the knife inserting the animal tissue at diagonal angles until the skeleton was reached. After the incision, the coral tissue contracted somewhat and after ∼5–10 min extrusion of mesenterial filaments, and tissue movement, often started coating sensor tips and interfering with the measurements. Therefore, measurement of each scalar irradiance profile commenced

immediately after the incision was made and was finished within ∼1–3 min. For each profile, the microsensor was carefully inserted into the cut until the skeleton surface was reached, by means of the micromanipulator and the automatic profiling function of the motor (step sizes of 50–100 μm). The position of the skeleton surface within the tissue was easily identified as a minimal retraction/bending of the optical fiber. Subsequently, profiling was done upward from the skeleton surface into the overlying tissue in steps of 100 μm logging the average of 10 spectra at each measuring depth until reaching the tissue surface (**Figure 1**).

To investigate the effect of changes in the ambient irradiance regime on the within tissue scalar irradiance we measured scalar irradiance within the polyp tissue (∼1000 μm depth) of *Platygyra lamellina* under increasing levels of downwelling photon irradiance spanning the range from 0 to 2000 μmol photons m^{-2} s^{-1}.

Raw spectra were integrated between (400–700 nm, PAR) and (700–800 nm, NIR) using the mathematical integration function of Origin Pro 8.0 (Origin, USA). Spectral profiles were normalized to E_d and smoothed if noisy, using the Savitzky–Golay function of Origin with a 50 point window; visual inspection of spectra before and after smoothing showed no loss of spectral details.

O_2 MICROSENSOR MEASUREMENTS

We used Clark-type O_2 microelectrodes with a tip size of 25 μm, a 90% response time of <0.5 s and a stirring sensitivity of ∼1% (Revsbech, 1989; OX25, Unisense AS, Denmark). Sensors were linearly calibrated against air saturated water and anoxic water (flushed with N_2). The percent air saturation in the seawater at experimental temperature and salinity was transformed to O_2 concentration (μmol O_2 L^{-1}) as described previously (Garcia and Gordon, 1992). The O_2 microsensor measurements inside the coral tissue were conducted using a similar approach as with the fiber-optic microprobes (see above). However, the O_2 microsensors approached the coral surface at an angle of ∼10° relative to the vertical as this facilitated better tissue penetration. For each measuring depth, we estimated steady state O_2 concentrations followed by gross photosynthesis estimates using the light–dark shift technique (see Revsbech and Jørgensen, 1983 for detailed description). Data were recorded with a conventional strip-chart recorder with a rapid time response (BD25, Kipp & Zonen, The Netherlands).

AUTHOR CONTRIBUTIONS

Daniel Wangpraseurt and Michael Kühl designed research; Daniel Wangpraseurt performed research; Anthony W. D. Larkum, Peter J. Ralph, and Michael Kühl contributed new reagents/analytic tools; Daniel Wangpraseurt, Anthony W. D. Larkum, and Michael Kühl analyzed data; and Daniel Wangpraseurt, Anthony W. D. Larkum, and Michael Kühl wrote the paper.

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Stimulated bacterioplankton growth and selection for certain bacterial taxa in the vicinity of the ctenophore *Mnemiopsis leidyi*

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Episodic blooms of voracious gelatinous zooplankton, such as the ctenophore *Mnemiopsis leidyi*, affect pools of inorganic nutrients and dissolved organic carbon by intensive grazing activities and mucus release. This will potentially influence bacterioplankton activity and community composition, at least at local scales; however, available studies on this are scarce. In the present study we examined effects of *M. leidyi* on bacterioplankton growth and composition in incubation experiments. Moreover, we examined community composition of bacteria associated with the surface and gut of *M. leidyi*. High release of ammonium and high bacterial growth was observed in the treatments with *M. leidyi* relative to controls. Deep 454 pyrosequencing of 16 S rRNA genes showed specific bacterial communities in treatments with *M. leidyi* as well as specific communities associated with *M. leidyi* tissue and gut. In particular, members of Flavobacteriaceae were associated with *M. leidyi*. Our study shows that *M. leidyi* influences bacterioplankton activity and community composition in the vicinity of the jellyfish. In particular during temporary aggregations of jellyfish, these local zones of high bacterial growth may contribute significantly to the spatial heterogeneity of bacterioplankton activity and community composition in the sea.

Keywords: *Mnemiopsis leidyi*, bacterioplankton, ctenophore, bacterial community composition

INTRODUCTION

In the past decade, the lobate ctenophore *Mnemiopsis leidyi*, which is native to the American east coast (Kremer, 1994), has spread to the Black and Caspian Seas (Finenko et al., 2006), the Mediterranean Sea (Shiganova et al., 2001), and more recently to the North Sea and the southern part of the Baltic Sea (e.g., Tendal et al., 2007). While it is well documented that this voracious predator (Colin et al., 2010) may graze upon a variety of plankton taxa (Granhag et al., 2011) and elicit cascading food web effects (Dinasquet et al., 2012), bottom-up effects associated with presence and activity of *M. leidyi* have received less attention.

Jellyfish may stimulate bacterioplankton growth by direct release of nutrients, ammonia in particular (Kremer, 1977), from tissue, mucus secretion, excretion, and sloppy feeding (reviewed in Pitt et al., 2009). High bacterial growth has been observed in the vicinity of decaying jellyfish (Titelman et al., 2006; Tinta et al., 2010); however, even more importantly, growth of bacterioplankton surrounding live jellyfish may be stimulated by the release of nutrients and bioavailable carbon (Condon et al., 2011). For instance, elevated bacterial growth was observed in a 0.5 m diameter zone around an *Aurelia aurita* medusa (Hansson and Norrman, 1995). Given that jellyfish have the potential to affect bacterioplankton growth, it is conceivable that they also affect bacterioplankton community composition. Release of bioavailable carbon would presumably select for specific bacterial groups, as recently observed using fluorescence *in situ* hybridization (Condon et al., 2011), but in addition release of specific bacterial taxa from colonized jellyfish tissue or from the jellyfish gut could affect composition of bacterioplankton in close proximity to the jellyfish, assuming that tissue or gut harbor specific communities. To what extent jellyfish are colonized by bacteria is to our knowledge not known; however, small crustaceans like copepods and cladocerans can host high densities of bacteria (Tang, 2005; Tang et al., 2010). Jellyfish, in contrast to crustacean zooplankton, do not change exoskeletons or molt during their life, but instead the same jellyfish surface decrease and increase during starvation and growth. Hence, it is conceivable that a microbiota inhabits surfaces of jellyfish. Consequently, as reported for e.g., non-gelatinous zooplankton (Grossart et al., 2010), fecal pellets (Jacobsen and Azam, 1984), or model aggregates (Kiørboe et al., 2003), a continuous exchange of bacteria between jellyfish surfaces and the surrounding water is conceivable.

In the present study we applied small-scale incubations with specimens of *M. leidyi* to examine effects on bacterioplankton growth and composition. Moreover, we examined community composition of bacteria associated with the surface and gut

of *M. leidyi*. Specifically, we aimed to test the hypotheses that (1) presence and feeding activity of *M. leidyi* affect bacterioplankton community structure and stimulate specific bacteria taxa in the vicinity of the jellyfish, and (2) bacteria attached to *M. leidyi* tissue or in the gut differ from those in the surrounding water.

MATERIALS AND METHODS

EXPERIMENTAL SET-UP

The experiment was initiated on 27 October 2010 at the Sven Lovén Centre for Marine Sciences in the Gullmar fjord at the west coast of Sweden (58° 15′N, 11° 27′E). At this time of the year only, *M. leidyi* is consistently present and performs local blooms (Friis-Møller, Tiselius; pers. comm.). Surface water, salinity 32, obtained from the fjord was 0.2 μm filtered (Supor filter, Pall), amended with 10% v/v 0.65 μm filtered (Millipore) fjord water (i.e., a bacterial inoculum), and 100 ml volumes were distributed in ten 100 mL acid-washed flasks, and manipulated in the following manner: three "control" treatments contained (1) water and inoculum as described above, (2) water and inoculum but with *M. leidyi* dipped into the water for 1 min to examine the potential immediate release of loosely associated bacteria from the *M. leidyi* tissue, and (3) water and inoculum with added copepods to examine potential release of bacteria from the copepods used as food items. Two additional treatments were amended with starved and fed *M. leidyi*, respectively. All treatments, in duplicates, were incubated for 41 h at 17°C under a 16:8 light:dark (h) cycle. Nutrients and bacterial community composition were measured at the start and end of the incubation, while bacterial abundance was measured six times (after 2, 13, 26, 37, and 41 h) during the course of the experiment. Nutrients and bacterial abundance were measured in the replicate flask. Since community composition was analyzed in only one replicate for each treatment, pseudoreplicates were used for ANOVA analysis of *M. leidyi* treatments (starved, fed, gut, and tissue) vs. controls.

CTENOPHORE AND COPEPOD TREATMENTS

The *M. leidyi* specimens were collected on 20 October 2010 with buckets from the surface in the Gullmar fjord and acclimatized in the laboratory for a week before the experiment. They were fed mixed zooplankton collected daily from the fjord using a 90 μm net. One day prior to the experiment feeding of ctenophores ceased, either without further addition of food (for starved *M. leidyi)* or with a final feeding with a few copepods (adult *Acartia sp.*) just before experiment start (for fed *M. leidyi*). At the beginning of the experiment, it was visually confirmed that *M. leidyi* specimens had empty guts (starved) or contained 3–4 visible copepods (fed). Each flask contained one ctenophore with an oral–aboral length of 10 mm. The *M. leidyi* specimens were continuously monitored. They were swimming and appeared in good condition throughout the whole experiment. The fed *M. leidyi* digested the copepods in their guts within ∼2 h.

Tissue (∼2 × 2 mm) from one of the dipped *M. leidyi* was aseptically dissected under a stereomicroscope from the ctenophore surface (mesoglea) and gut (pharynx) and stored frozen in sterile Eppendorf tubes at −80°C until DNA extraction. The specimen was treated in the same way as the starved animals and was not fed for 24 h prior to the dissection. A starved specimen was chosen in order not to include microbiota associated with prey in the gut analysis.

In the zooplankton incubation, three copepods (adult *Acartia* sp.) were added per flask and they remained in the flask throughout the incubation. The copepods were from the same zooplankton tow as was used in the final feeding of *M. leidyi* before the experiment.

MEASUREMENTS OF INORGANIC NUTRIENTS AND BACTERIAL ABUNDANCE

At termination of the experiment 10 mL water from each treatment were 0.45 μm filtered (Millipore) and frozen at −20°C. The samples were thawed and analyzed for ammonium, nitrate, and nitrite as well as phosphate with a TRAACS 2000 (Bran + Luebbe). For nutrients one-factor ANOVAs were conducted to test for differences between control and *M. leidyi* treatments. Samples (1.5 mL) for bacterial enumeration were fixed with EM grade glutaraldehyde (Sigma; 1% final conc.), frozen in liquid N_2 and stored at −80°C. Cells were stained with SYTO 13 (Molecular Probes) and counted on a FACSCalibur flow cytometer (Becton Dickinson) according to Gasol and del Giorgio (2000). Fluorescent beads (True counts, Becton Dickinson) were used to calibrate the flow rate. Replicate counts of the same sample generally varied less than 5%. For bacterial abundance one-factor ANOVA was used to test for differences between the five treatments (pooling data from time 37 and 41) and Student-Newman-Keuls (SNK) post hoc test to determine between which treatments differences occurred. A Type I error rate of 0.05 was used.

BACTERIAL COMMUNITY COMPOSITION

Bacterial community composition was analyzed in a total of eight samples: six water samples (one replicate from the initial water and from each treatment after incubation), one *M. leidyi* tissue sample, and one *M. leidyi* gut sample. Water samples (80 mL) were filtered onto 0.2 μm sterivex filters (Millipore), which were then frozen at −80°C in 1 mL sucrose lysis buffer (20% sucrose, 50 mM EDTA, 50 mM TrisHCl, pH = 8). DNA was extracted from the sterivex filters using an enzyme/phenol-chloroform protocol (Riemann et al., 2000) but with a 30-min lysozyme digestion at 37°C and an overnight proteinase K digestion (20 mg ml^{-1} final conc.) at 55°C (Boström et al., 2004). DNA from *M. leidyi* tissue and gut samples was extracted using the EZNA tissue DNA kit (Omega Bio-Tek). DNA was quantified using Nanodrop (Thermo Scientific). Bacterial 16 S rRNA genes were PCR amplified using puReTaq Ready-To-Go PCR beads (GE Healthcare), 0.06 ng DNA μl^{-1}, and primers 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 805R (5′-GAC TAC HVG GGT ATC TAA TCC-3′). The amplification was run in two steps: a first step with the regular primers (20 cycles), followed by re-amplification of 1 μl product using the same primers complemented with 454-adapters and sample-specific barcodes (five cycles; Berry et al., 2011). For each sample, triplicate PCR products from independent runs were pooled prior to purification (Agencourt AMPure XP kit, Beckman Coulter) and quantification (PicoGreen, Molecular Probes). The samples were mixed in equimolar amounts and

sequenced from the reverse primer direction using Roche/454 GS FLX Titanium technology (National High-throughput DNA Sequencing Centre, University of Copenhagen).

PHYLOGENETIC ANALYSIS

Sequences were analyzed and processed using the Quantitative Insights Into Microbial Ecology software (QIIME v1.4; Caporaso et al., 2010b) with default settings, excluding sequences <350 bp or >450 bp. Flowgrams were denoised directly in the pipeline (Reeder and Knight, 2010). All singletons were removed. Sequences were then clustered into operational taxonomic units (OTUs) at 97% pairwise identity using the seed-based Uclust algorithm, and representative sequences from each OTU aligned to the Greengenes imputed core reference alignment (DeSantis et al., 2006) (http://greengenes.lbl.gov) using PyNAST (Caporaso et al., 2010a). Chimeras were removed using Chimera Slayer (Haas et al., 2011). Taxonomy assignments were made using the ribosomal database project (RDP) classifier (Wang et al., 2007). Sequences have been deposited in the CAMERA database (Community Cyberinfracstructure for advanced microbial ecology research and analysis; http://camera.calit2.net/index.shtm) under accession number CAM_P_0000918. The phylogenetic similarity between samples was determined with the unweighted pair group method using an arithmetic (UPGMA) mean tree calculated from the jack-knife-weighted UniFrac distance matrix within QIIME. The matrix was calculated with randomly picked OTUs (normalized to 1043 sequences per sample to accommodate for the lowest number of sequences found in a sample). The random subsampling was done within QIIME. In order to present a succinct overview of the main taxa in the samples, OTUs summing to >100 sequences across all samples were used for further log 10 + 1 transformation and visualization in a heatmap. The heatmap was made using the CIMminer tool for clustered image maps (http://discover.nci.nih.gov/).

RESULTS AND DISCUSSION

NUTRIENT CONCENTRATIONS AND BACTERIAL GROWTH

Release of ammonium in the *M. leidyi* treatments (starved and fed) was higher than in control treatments (one factor ANOVA $F_{(1, 8)} = 89$, $p = 0.0001$; **Table 1**). The ammonium release was more variable in fed *M. leidyi*, which may be due to variations in digestion time and start of starvation between specimens. For the starved *M. leidyi*, ammonium release within the two replicates had similar values. The ammonium release was expected to be higher in starved animals as ammonium is an end product during starvation, which initiates within few hours without food (Kremer and Reeve, 1989). This excretion is dependent on the feeding history of the ctenophores (Kremer, 1982; Kremer et al., 1986) as well as on temperature, affecting the digestion process and thereby the excretion rate (Kremer, 1977; Nemazie et al., 1993). In our case the ammonium release was calculated to be 15 µmol ammonium g DW ctenophore^{-1} day^{-1} (assuming 10 mm oral-aboral length equals 0.04 g DW; Friis-Möller unpublished data), which is within the ammonium release rates commonly found for gelatinous zooplankton (reviewed by Schneider, 1990). Phosphate-levels were also higher in *M. leidyi* treatments than in control treatments (one factor ANOVA $F_{(1, 8)} = 21.8$, $p = 0.0016$) but for nitrite/nitrate no difference were seen (one factor ANOVA $F_{(1, 8)} = 0.36$, $p = 0.563$).

Similar to the ammonium and phosphate levels, bacterial abundance was higher in the *M. leidyi* treatments after 37 h of incubation compared to control treatments (one factor ANOVA $F_{(4, 15)} = 12.41$, $p = 0.0001$). With the SNK post-hoc test the bacterial abundance was seen to differ between starved and fed *M. leidyi* but there were no differences between the water treatment control, the treatment with dipped *M. leidyi* or the treatment with copepods (**Figure 1**).

The observed responses were anticipated since ctenophores are known to release large amounts of ammonium (Pitt et al., 2009; and references therein). In addition, in combination with the release of bioavailable carbon (Condon et al., 2011), a stimulated bacterial growth was expected (Church, 2008). Interestingly, the observation of extensive bacterioplankton growth associated with both fed and starved *M. leidyi* points to an important

Table 1 | Concentrations of inorganic nitrogen and phosphorus (µmol L^{-1}) at the start of the experiment and at the end in the different treatments.

	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}
Start water	1.5	<0.1	0.5
Control water	0.78	3.21	1.32
	0.67	3.09	1.06
Water with dipped *M. leidyi*	0.55	3.00	0.82
	0.43	3.17	1.15
Water with copepods	0.90	3.30	0.98
	0.88	3.23	0.69
Water with fed *M. leidyi*	7.96	3.20	2.30
	15.18	3.21	2.44
Water with starved *M. leidyi*	13.17	3.22	1.84
	13.84	3.17	1.44

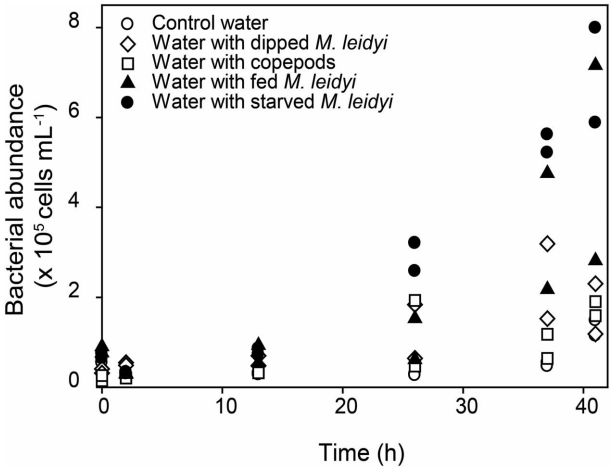

FIGURE 1 | Changes in bacterial abundance over time. White symbols represent control treatments and black symbols represent treatments with *M. leidyi*. Data from both replicates are shown for all treatments.

role for carbon and nutrients released directly from *M. leidyi* tissue.

BACTERIAL COMMUNITY COMPOSITION

Using 454-pyrosequencing we sought to test the hypotheses that (1) presence and activity of *M. leidyi* stimulate specific bacteria taxa, and (2) that bacteria attached to *M. leidyi* tissue or in the gut differ from those in the surrounding water. A total of 32,736 partial 16S rRNA gene sequences remained after quality controls, yielding on average 4022 reads per sample (range 1420–7770 reads) and a total of 364 unique OTUs in the whole dataset. The rarefaction curves showed saturation for all samples, except for the start water and for the *M. leidyi* gut (data not shown). Hence, our sequencing effort did not cover bacterial diversity well in these two samples.

Comparisons based on weighted-UniFrac distances between the sub-sampled datasets showed that bacterial communities in waters with fed and starved *M. leidyi* were relatively similar to the community associated with *M. leidyi* tissue (**Figure 2**). Likewise, the three control treatments were found in one cluster. Dipping of *M. leidyi* for 1 min had a relatively minor effect on bacterial community composition in the surrounding water indicating that a large pulsed release of bacteria loosely associated with the tissue did not take place. The lack of a community response to the presence of copepods was surprising to us because copepods are often heavily colonized by bacteria that may exchange with surrounding water (Møller et al., 2007), and a high concentration of copepods was used relative to *in situ* conditions. The bacterial communities in the initial water and in the *M. leidyi* gut were very different from the other samples. These clustering patterns indicate that presence and/or activity of *M. leidyi* has a distinct effect on bacterioplankton in the surrounding water.

Community composition at the phylum level (phylum and proteobacterial subclass, hereafter referred to as phylum) varied between treatments (**Figure 3**). The start water was dominated by α-proteobacteria followed by Actinobacteria, γ-proteobacteria, and Bacteroidetes. These phyla are commonly predominant in Skagerrak (Pinhassi et al., 2003; Sjöstedt et al., 2012). After 41 h of incubation, the control treatments were dominated by γ-proteobacteria (**Figures 3A–C**). Similarly, treatments with starved and fed *M. leidyi* were also dominated by γ-proteobacteria (**Figures 3D,E**); however, with a much higher contribution of Bacteroidetes (tested in one factor ANOVA $F_{(1,6)} = 8.32$, $p = 0.028$). Interestingly, proliferation of γ-proteobacteria and Bacteroidetes in the vicinity of *M. leidyi* specimens was also recently demonstrated using fluorescence *in situ* hybridization (Condon et al., 2011). This may indicate niche partitioning defined by the nutrient field in the vicinity of *M. leidyi*; however, the selective drivers causing this bacterial succession remain to be identified.

COMPOSITION OF BACTERIA ASSOCIATED WITH *M. leidyi* TISSUE AND GUT

The gut community was different from the community present on the tissue, and was mainly dominated by Bacteroidetes and α-proteobacteria but also contained Cyanobacteria and Actinobacteria (**Figures 3F,G**). The dominant OTUs associated with *M. leidyi* (gut, tissue or surrounding water) were related to Flavobacteriaceae (Bacteroidetes) and Rhodobacteraceae (α-proteobacteria); these groups were especially prominent in the gut (**Figure 4**). *Roseobacter* strains (member of the Rhodobacteraceae family) have been shown to colonize marine algae and dinoflagellates (reviewed in Slightom and Buchan, 2009), and copepods in the North Sea (Møller et al., 2007). This may indicate that some bacterial taxa detected in the gut originate from prey. Based on the approach applied here it is unfortunately not possible to determine to what extent the detected gut sequences originate from bacteria associated with prey, colonizing free-living bacteria, or from a more permanent symbiotic gut microflora. However, since the dissected animal was starved for

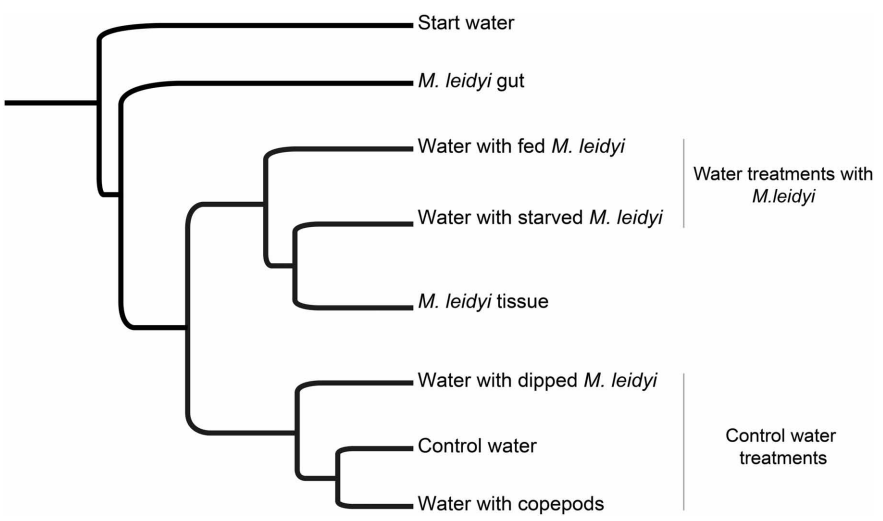

FIGURE 2 | UPGMA tree calculated from the Jackknife-weighted UniFrac distance matrix, displaying the phylogenetic distances between samples (based on the subsampled datasets of 1043 sequences).

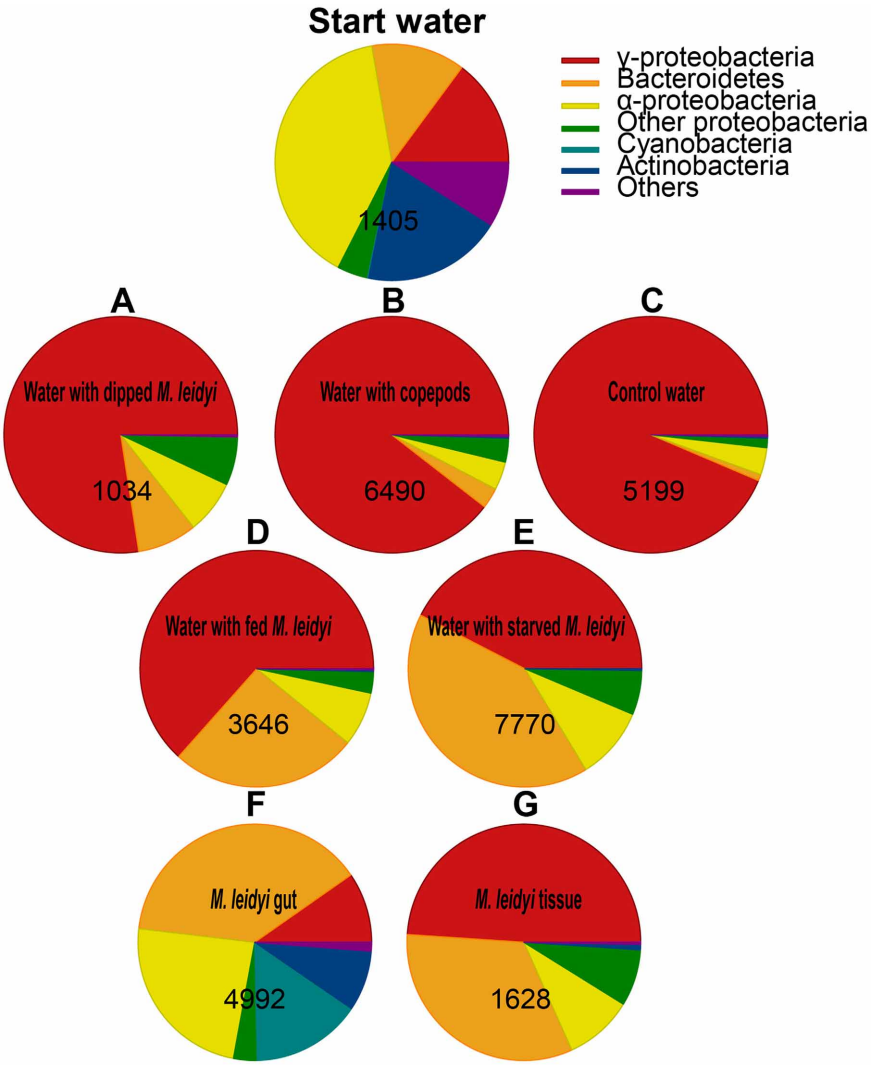

FIGURE 3 | Relative abundance of the major phyla and proteobacterial sub-classes expressed as the % of total sequences obtained from the sample. Numbers indicate the total number of sequences per sample. "Others" represent phyla with <1% of relative abundance. Control treatments: water with dipped *M. leidyi* **(A)** water with copepods **(B)** and control water **(C)**. Water treatments with *M. leidyi*: fed **(D)** and starved **(E)**. *M. leidyi* gut **(F)** and tissue **(G)**.

24 h prior to dissection, and prey was not visible in the gut, it is likely that the acquired sequences originate from the gut rather than from prey items *per se*.

FLAVOBACTERIACEAE ASSOCIATED WITH *M. leidyi*

Bacterial community composition associated with *M. leidyi* (water, tissue, and gut) showed a high prevalence of Bacteroidetes (26–41% of the sequences) relative to the control treatments (1–7% of the sequences; tested in one factor ANOVA $F_{(1,6)} = 8.32$, $p = 0.028$; **Figure 3**); in particular, members of the Flavobacteriaceae were prominent, especially in the gut (**Figure 4**). Flavobacteria are known to be enriched on organic particles and show a strong capacity for degrading complex polymers (Kirchman, 2002). Moreover, Flavobacteriaceae produce chitin degrading enzymes and enzymes used for the degradation of algae (reviewed in Bernardet and Nakagawa, 2006) and have recently been reported to play an important role for the degradation of jellyfish tissue (Tinta et al., 2012). Hence, these bacteria conceivably have the metabolic capacity to facility prey digestion in the *M. leidyi* gut as well as to colonize and degrade tissue.

Two main Flavobacterium OTUs were related to the genus *Tenacibaculum* (**Figure 4**), containing the fish pathogen *Tenacibaculum maritimum*, an agent of the gill disease tenacibaculosis (Handlinger et al., 1997). This bacterium, which has also been reported from two other jellyfish species (Ferguson et al., 2010; Delannoy et al., 2011), produces highly proteolytic enzymes (Bernardet and Nakagawa, 2006) and could play a role in the jellyfish digestive metabolism or, in concert, in degradation of jellyfish tissue. It has been suggested that jellyfish could act as vectors for this pathogen in fish aquacultures (Ferguson et al., 2010; Delannoy et al., 2011). Indeed, the repeated findings, including ours, of the *Tenacibaculum* genus associated with

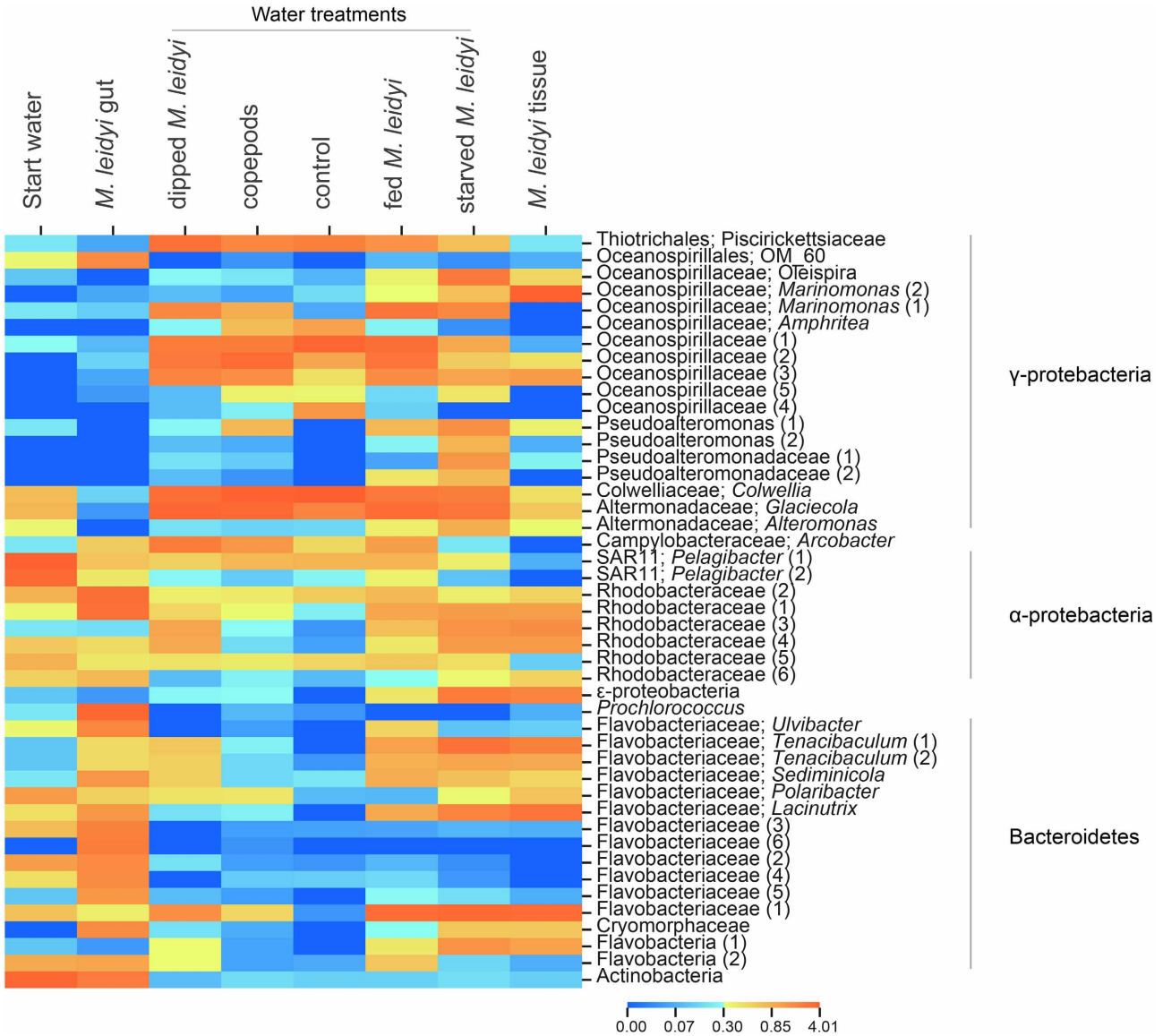

FIGURE 4 | Heatmap displaying the relative abundances of specific OTUs across the samples. Only OTUs with a sum of >100 assigned sequences across all samples were used. The color scale represents the log10+1 transformed relative abundance of the number of sequences. Duplicate OTUs were numbered sequentially for clarification.

jellyfish is noteworthy; however, additional information on the genetic and functional resemblance of jellyfish associated bacteria and *Tenacibaculum maritimum* is necessary to establish the distribution and ecology of this pathogen in gelatinous plankton.

CONCLUDING REMARKS

The present study suggests that *M. leidyi* from the Gullmar fjord in autumn harbors specific bacterial communities in the gut and tissue, and that the presence and activity of *M. leidyi* influences bacterioplankton activity and community composition in the vicinity of the jellyfish. Comparisons of community composition in the individual treatments should, however, be interpreted with caution, since they are based on sequencing of single samples from small bottle incubations. While the bacterial growth in replicate incubations in most cases yield confidence in a corresponding similar compositional succession, extensive sequencing of true replicate samples would be needed to draw firm conclusions on community composition in specific treatments. The high reproducibility of 454 pyrosequencing gives, however, some confidence in the obtained results. For instance, a phylum-level standard deviation of less than 2% of read abundance between technical replicates was recently shown for indigenous bacterial communities in sediments (Pilloni et al., 2012). Finally, the strong selection for Flavobacteriaceae was supported by our data from the gut, tissue, and the treatments with *M. leidyi*. This finding as well as the stimulation of bacterial growth in the

vicinity of the ctenophore may have several implications: (1) Due to the specific bacterial communities associated with *M. leidyi*, it may be speculated that it can serve as a vector for relocation of microbial species between pelagic environments, a phenomenon demonstrated for zooplankton (Grossart et al., 2010); (2) Our observations point to strong local bottom-up effects associated with *M. leidyi*. Hence, in addition to food web interactions elicited by jellyfish grazing (Pitt et al., 2009), enhanced bacterioplankton growth in the vicinity of the jellyfish could contribute to the spatial heterogeneity of bacterioplankton growth and community composition in the sea. This could be particularly important during ctenophore blooms (like in autumn in the Gullmar fjord) or in relation to physical gradients (like for example temperature and salinity), physical discontinuities (like fronts) or surface features like Langmuir cell circulations where temporary aggregations of gelatinous plankton occur (Graham et al., 2001). Within such hydrodynamic boundaries, ctenophores, and consequently elevated nutrient levels, could be retained, leading to local changes in bacterioplankton activity and community composition. For instance, a selection for Flavobacteriaceae, with their ability to degrade complex polymers (Kirchman, 2002), could affect the distribution and fluxes of nutrients and carbon, ultimately affecting dynamics of primary production and higher trophic levels.

Along with other recent studies (e.g., Pitt et al., 2009; Condon et al., 2011; Tinta et al., 2012), the present work contributes to the emerging picture of extensive and dynamic interactions between gelatinous plankton (live and dead) and bacteria, which has local consequences for bacterial activity and community composition, and likely influence jellyfish ecology. A deeper mechanistic understanding of the extent and spatio-temporal variability is, however, needed in order to establish the ecological implications of jellyfish—bacteria interactions in marine waters.

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Free-living and plankton-associated vibrios: assessment in ballast water, harbor areas, and coastal ecosystems in Brazil

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Ballast water (BW) is a major transport vector of exotic aquatic species and pathogenic microorganisms. The wide-ranging spread of toxigenic *Vibrio cholerae* O1 from harbor areas has been frequently ascribed to discharge of contaminated BW into eutrophic coastal environments, such as during the onset of the seventh cholera pandemic in South America in the early 1990s. To determine the microbiological hazards of BWs transported to Brazilian ports, we evaluated water and plankton samples taken from (i) BW tanks of recently arrived ships, (ii) port areas along the Brazilian coastline from ∼1 to 32˚S and (iii) three coastal areas in São Paulo State. *Vibrio* concentration and toxigenic *V. cholerae* O1 occurrence were analyzed. Plankton-associated vibrios were more abundant than free-living vibrios in all studied environments. *V. cholerae* was found in 9.5% of ballast tanks and 24.2% of port samples, both as free-living and attached forms and, apart from the Santos harbor, was absent off São Paulo State. Toxigenic *V. cholerae* O1 isolates (*ctx*A$^+$, *tcp*A$^+$), involved in cholera disease, were found in BW (2%) and harbor (2%) samples. These results confirm that BW is an important carrier of pathogenic organisms, and that monitoring of vibrios and other plankton-attached bacteria is of paramount importance in BW management programs.

Keywords: plankton-vibrio symbiosis, ballast water, port areas, bacterial hazard, *Vibrio cholerae*

INTRODUCTION

Ballast water has been considered a principal transport vector of aquatic species of plants, animals, and microorganisms across biogeographic provinces, and to cause major changes in the composition and function of ecological communities in freshwater, estuarine, and marine ecosystems (Williams et al., 1988; Carlton and Geller, 1993; Ruiz et al., 2000). Ballast capacity varies according to vessel size and cargo type. A large commercial ship may carry an excess of 200,000 m^3 of ballast water (BW), and discharge rates are as high as 20,000 $m^3\ h^{-1}$ (NRC, 1996). Consequently, a large quantity and diversity of planktonic and benthic species occur in ballast tanks. Such species may succeed in transposing natural biogeographic barriers when viable individuals are released in the new environment through BW discharge, either in a single introduction event or as repeated inoculations (Williams et al., 1988; Carlton and Geller, 1993).

Bacteria belonging to the *Vibrionaceae* family, the so-called vibrios, are autochthonous from aquatic ecosystems worldwide and commonly found both as free-living cells and in association with plankton (Simidu et al., 1971; Kaneko and Colwell, 1975). Since the observation of *Vibrio cholerae* attached to copepod egg sacs and mouthparts (Huq et al., 1983), many studies have shown that vibrios are symbiotic to a wide range of zooplankton taxa (Louis et al., 2003; Rawlings et al., 2007; Lizarraga-Partida et al., 2009; Turner et al., 2009; Martinelli Filho et al., 2010).

Strong evidence exists that cargo ships are important transport vectors of cholera and other vibrio-related diseases (McCarthy and Khambaty, 1994; Ruiz et al., 2000; Drake et al., 2005, 2007; Mimura et al., 2005). Contaminated BW discharge into harbor and coastal waters could increase the likelihood of local horizontal gene transfer between toxigenic and non-toxigenic vibrio strains (Chiang and Mekalanos, 1999), thus setting the conditions for the spread of diarrheic outbreaks into a new location. This demands particular attention in coastal areas affected by impaired sanitary conditions, as in the case of many developing countries (Rivera et al., 2008). In addition, there is indication that anthropogenic climate change is driving the emergence of *Vibrio* disease in temperate regions (Baker-Austin et al., 2012). As a consequence, the spread of *Vibrio* species by maritime transport becomes a matter of public health concern even in countries where coastal pollution is a relatively minor environmental problem.

A significant percentage of cargo loads in Brazil is due to oil tankers and bulk carriers, which account for most of the BW transported globally. Loads moved by Brazilian ports and private terminals have more than doubled since the early 1990s, as a consequence of the country's economical growth, and a corresponding increase in BW discharge by ocean-going ships is most likely underway (Oliveira, 2008). Our study covered the largest Brazilian harbors and some of the busiest coastal regions in terms of maritime transport.

We expand here the existing information on vibrios and *V. cholerae* prevalence in BW, harbor areas, and coastal regions, showing that toxigenic *V. cholerae* O1 occurs in ballast tanks, and that plankton-associated vibrios are two to four orders of magnitude

more abundant than free-living vibrios, both in BW tanks and in the marine environment.

MATERIALS AND METHODS

BALLAST WATER AND PLANKTON SAMPLES FROM SHIPS ARRIVING TO BRAZILIAN PORTS

Fifteen ports (**Figure 1**) were selected in nine Brazilian states based on their geographical representativeness along the extensive Brazilian coast, and according to operational characteristics such as prevailing navigation routes of arriving ships, ship types, and ship traffic volume. One hundred five commercial vessels were sampled covering both international and domestic routes (80 and 20% of samples, respectively). Using a suction pump, BW samples were collected from upper wing, fore peak, or double bottom tanks (one tank per vessel) accessed through sounding pipes, ellipses, scuttles, or vent pipes. One liter of BW was transferred to a sterile plastic bottle, after flushing at least 100 L of water from the pump hose. Pump pipes were abundantly rinsed and emptied after each use. Plankton samples were collected with the same pump by filtering 50–400 L of BW by wet-sieving through a 100-μm meshed-size conical net. Plankton samples were also transferred to 250 mL sterile plastic bottles. Sampling was done from October 2001 to 2002.

WATER AND PLANKTON SAMPLES FROM BRAZILIAN HARBOR (H) AND COASTAL AREAS

Seven Brazilian harbor areas were included in this study (**Figure 1**), each with six sampling stations along the harbor area and positioned from 40 m to 1 km off the main pier. Ninety water and 90 plankton samples were collected from October 2002 to April 2003 (**Figure 1**).

Three coastal regions in São Paulo state were additionally studied: Santos region (three stations), São Sebastião Channel (SSC; two stations), and Ubatuba (two stations; **Figure 1**). These stations/sites were selected according to their trophic status and level of anthropogenic influence. For instance, Santos is more urbanized and eutrophic than São Sebastião and Ubatuba (Burbano-Rosero et al., 2011).

Five liters of water were collected in a sterile plastic bottle during high tide for each station. Plankton samples were collected at the same locations by subsurface horizontal tows of a 64 μm mesh-sized net and transferred to a 250 mL sterile plastic container. In São Sebastião Channel, samples were collected monthly from August 2005 to March 2007 (20 months), while in Santos and Ubatuba sampling was performed during 2006 and 2007 summer seasons. A total of 32 seawater and 32 plankton samples were collected in São Sebastião; 15 seawater and 15 plankton samples in Santos; and 8 seawater and 8 plankton samples in Ubatuba (**Figure 1**).

ENVIRONMENTAL DATA

Salinity and temperature were measured in BW and environmental samples using a portable multi-probe (Hach Company). In order to summarize the salinity data, the water samples were classified in four categories: (1) oligohaline (less than 5 psu), poly/mesohaline (5–30 psu), euryhaline (30–35 psu), and oceanic (more than 35 psu).

We emphasized salinity as a main environmental variable in this study because ballast discharge regulations in Brazil are currently based on open-ocean exchange, which is validated by salinity inspection under the responsibility of port state control authorities.

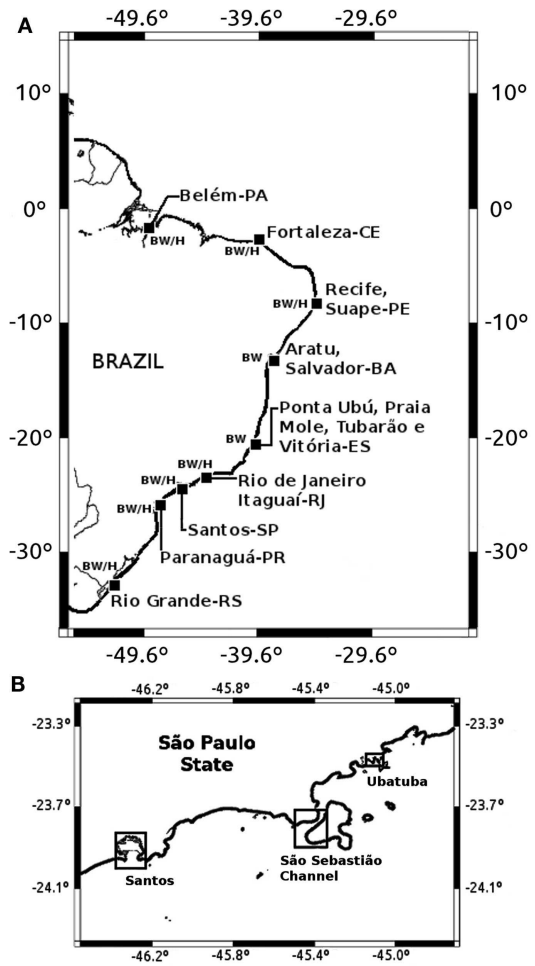

FIGURE 1 | Locations of ballast water (BW) and environmental (H) sampling sites in Brazilian ports (A) and in São Paulo State coastal areas (B).

Because sampling was performed in different periods of time between 2001 and 2007 for BW, harbor areas, and coastal regions of São Paulo state, correlations between vibrios and environmental data were performed separately according to the sample source. Zooplankton composition and biomass, as well as chlorophyll and other proxies for coastal eutrophication were not analyzed in this study.

SAMPLE TRANSPORT TO THE LABORATORY

Immediately after sampling water and plankton samples were stored in an insulated container at 4°C and dispatched by courier or carried to the Environmental Microbiology Lab in São Paulo. Total time from sampling to analysis did not exceed 24 h.

MARINE VIBRIOS COUNTING

Concentration of viable vibrios was obtained with the plate count method associated with the Simidu and Tsukamoto (1980)

medium. For plankton samples, 1 g (wet weight) was ground and three dilutions (1/10) were serially performed. The plates were incubated at 20°C for 72 h in anaerobic conditions (Anaerobac, Probac, SP, Brazil). *Vibrio* concentration was expressed as colony-forming units (CFU) mL^{-1} and CFU g^{-1} for water and plankton samples, respectively. Cultural determinations were based on duplicate plates at a given dilution, and results expressed as means. Considering that typical zooplankters such as calanoid copepods have a mean mass density only slightly higher than that of seawater (e.g., from 1.0274 to 1.0452 g cm^{-3} for *C. finmarchicus*; Knutsen et al., 2001), a direct comparison between free-living and attached bacterial concentrations in volumetric and mass terms is realistic for the purposes of this study.

VIBRIO CHOLERAE DETECTION AND CHARACTERIZATION

The enrichment method was used and water samples (100 mL) were filtered through 0.22 μm nitrocellulose membrane filters (Millipore), inoculated onto 25 mL of Alkaline Peptone Water (APW; 1% peptone; 1% NaCl; pH 8.4) and incubated at 30°C for 12–16 h. For plankton samples, 1 g (wet weight) was crushed, inoculated into 25 mL of APW and incubated under the same conditions. Two loops of the enrichment were streaked on thiosulfate-citrate-bile salts – TCBS Agar (Oxoid) and after incubation at 30°C for 18–24 h about five characteristic yellow colonies were transferred to Luria Agar (Difco). These colonies were screened for *V. cholerae* by the presumptive oxidase and string tests. The positive strains were preliminary screened by a short biochemical series (Choopun et al., 2002) and identified as *V. cholerae* by PCR (Chun et al., 1999). *V. cholerae* strains were serotyped by antiserum for O1 and O139 serogroups (Probac do Brasil). The serogroup was then confirmed by a multiplex PCR (Rivera et al., 2003). The detection of *ctx*A and *tcp*A genes, associated to virulence, was carried out by multiplex PCR using 94F and 614R primers for *ctx*A gene (Fields et al., 1992) and the 72F and 477R primers for *tcp*A gene (Rivera et al., 2001).

RESULTS

BALLAST WATER TANKS

Free-living viable vibrios were more frequent (32%) than plankton-associated vibrios (18%) in BW samples but were not as abundant. Plankton-associated vibrios varied from <10 to 5,100 CFU g^{-1} while free-living vibrios varied from <1 to 430 CFU mL^{-1}. Mean vibrio counts did not change significantly across the wide salinity gradient observed in BW tanks (Kruskal–Wallis test, $p > 0.05$). The temperature of BW samples varied from 19 to 35°C (mean 25.8°C) and the salinity varied widely, from 0.1 to 39.5 psu (mean 30.3).

Vibrio cholerae was detected in 13.3% of water and 5.7% of plankton samples from ballast tanks. *V. cholerae* O1 was isolated from eleven ballast tanks collected from ships arriving in Belém, Fortaleza, Recife, Santos, Itaguaí, Paranaguá, Ponta Ubú, and Rio Grande harbors (**Table 1**). Toxigenic *V. cholerae* O1, genotype *ctx*A^+/*tcp*A^+, was found in one water sample from a ship arriving in Belém and in one plankton sample from a ship arriving in Recife port. Two other BW samples contained *V. cholerae* O1, genotype *ctx*A^+/*tcp*A^-. Interestingly, *V. cholerae* non-O1 genotypes *ctx*A^+/*tcp*A^+ and *ctx*A^+/*tcp*A^- were found in four other BW samples (**Table 1**).

BRAZILIAN HARBORS AREAS (H)

Vibrios were prevalent in Brazilian harbor areas both as free-living (97%) and plankton-associated (96%) forms. Plankton-associated vibrios were more abundant (up to 2.4×10^6 CFU g^{-1}) than free-living (up to 4.4×10^3 CFU mL^{-1}; **Table 2**), and no relationship with salinity (Kruskal–Wallis test, $p > 0.05$) was observed in neither case (**Figure 2**). Temperature of harbor water samples varied from 16 to 35°C and salinity varied from 0.1 to 36.3 psu. The salinity values at the ports of Belém (PA) and Rio Grande (RS) were typical of freshwater and the remaining ports had estuarine characteristics.

Both free-living and plankton-associated *V. cholerae* occurred in all harbor areas except Fortaleza and Rio Grande. Toxigenic *V. cholerae* O1 (*ctx*A^+/*tcp*A^+) isolates were found in water samples collected at Recife and Paranaguá, and in plankton samples collected at Santos harbor areas. Toxigenic *V. cholerae* non-O1 (*ctx*A^+/*tcp*A^+) was found as free-living forms in Belém, Recife, Santos, and Paranaguá harbors, and associated to plankton in the Recife and Santos harbor areas (**Table 3**).

COASTAL SITES OFF SÃO PAULO

Mean plankton-associated viable vibrio abundance was again higher than free-living forms by two to four orders of magnitude (**Table 4**). Counts were higher for free-living vibrios in lower salinities ($p < 0.001$) but no difference occurred in the case of plankton-associated vibrios ($p > 0.1$). Mean free-living vibrio concentration was higher in Santos ($p < 0.001$) when compared with São Sebastião Channel and Ubatuba (**Figure 3**).

Vibrio cholerae was not detected in coastal water or plankton samples with the traditional enrichment method applied here. From 110 collected samples, only 50 suspected colonies were obtained. However, when submitted to biochemical and molecular assays, those isolates were not confirmed as *V. cholerae*.

DISCUSSION

Cargo operations have a direct impact on BW management by commercial vessels, as BW is discharged to match a proportional amount of cargo being loaded. Using correlational methods based on such postulation, Medeiros (2004), Clarke et al. (2004), and Oliveira (2008) have shown that Brazilian harbors included in this study are recipients of BW imported from other biogeographic provinces.

Bacteria are known to attain high growth rates when associated to either live zooplankton or their carcasses, exuvia, and fecal pellets, as these substrates provide a much richer organic medium than the surrounding environment (Tang et al., 2010). Our results confirm such trend, as plankton-associated vibrios were two to four orders of magnitude more abundant than free-living bacteria in BW tanks, harbor areas, and coastal sites.

While associated vibrios were common in all aquatic ecosystems analyzed (>95% of samples), a much lower proportion of BW tanks contained either associated or free-living vibrios (18 and 32% of samples, respectively). Such low prevalence compared to the natural environment is probably related to increased zooplankton mortality in BW tanks (Gollasch et al., 2000) and the

Table 1 | Characteristics of *V. cholerae* strains isolated from ballast water tanks of ships arriving in Brazilian ports, with temperature and salinity records.

Sample sequence and port identification	Ballast water		*Vibrio cholerae*		
	Temperature (°C)	Salinity (psu)	Free-living (F) or plankton-associated (A)	Serogroup	Genotype*
Belém	30	4.8	F	O1	*ctx*A$^+$/*tcp*A$^-$
Belém	32	29.8	F	O1	*ctx*A$^+$/*tcp*A$^+$
Belém	30	7.7	F	Non-O1	*ctx*A$^+$/*tcp*A$^+$
Fortaleza	28	9.7	F	O1	*ctx*A$^-$/*tcp*A$^-$
Recife	32	36.9	F	Non-O1	*ctx*A$^+$/*tcp*A$^-$
				O1	*ctx*A$^+$/*tcp*A$^-$
			A	O1	*ctx*A$^+$/*tcp*A$^+$
Recife	28	35.4	F	O1	*ctx*A$^-$/*tcp*A$^-$
			A	Non-O1	*ctx*A$^-$/*tcp*A$^-$
Ponta Ubu	20	34.4	F	O1	*ctx*A$^+$/*tcp*A$^-$
Ponta Ubu	22	34.6	F	Non-O1	*ctx*A$^+$/*tcp*A$^+$
Itaguaí	ND	34.3	F	Non-O1	*ctx*A$^+$/*tcp*A$^-$
Itaguaí	25	34.5	F	O1	*ctx*A$^-$/*tcp*A$^-$
Santos	29	35.3	F	O1	*ctx*A$^-$/*tcp*A$^-$
Santos	27	34.0	F	O1	*ctx*A$^-$/*tcp*A$^-$
			A	O1	*ctx*A$^-$/*tcp*A$^-$
Santos	27	34.5	F	O1	*ctx*A$^-$/*tcp*A$^-$
			A	O1	*ctx*A$^-$/*tcp*A$^-$
Santos	22	35.8	F	Non-O1	*ctx*A$^+$/*tcp*A$^+$
Paranaguá	26	34.9	A	O1	*ctx*A$^-$/*tcp*A$^-$
Rio Grande	19	33.4	A	Non-O1	*ctx*A$^-$/*tcp*A$^-$

**Genotypes ctxA and tcpA are genes codifying for cholera toxin and toxin co-regulated pilus, respectively. ND, not determined.*

Table 2 | Temperature, salinity, total coliforms, and vibrio frequency and concentration in environmental samples collected in Brazilian harbor areas.

Ports	*n*	Temperature (°C)		Salinity		Total coliforms (CFU mL^{-1})		Free-living vibrios (CFU mL^{-1})			Plankton-associated vibrios (CFU g^{-1})		
		Min	Max	Min	Max	Min	Max	%	Min	Max	%	Min	Max
Belém	6	29	30	0	0.1	400	$>2.0 \times 10^4$	100	2.8×10^2	1.6×10^3	100	1.4×10^3	8.6×10^4
Fortaleza	6	27	27	27.3	35.9	<1	1.6×10^3	100	2.4×10^1	1.8×10^2	100	1.7×10^3	1.6×10^4
Recife	24	23	29	26.2	34.9	<1	2.0×10^2	100	4.2×10^1	4.4×10^3	91.7	<10	3.0×10^5
Santos	24	21	27	10.8	32.5	<1	4.1×10^2	96.7	<1	1.1×10^3	100	2.0×10^2	$1.6 \times 10^6 \psi$
Paranaguá	18	16	35	16.8	27.4	16	9.2×10^2	100	1.5×10^1	3.3×10^3	94.4	1.2×10^3	$2.4 \times 10^6 \psi$
Itaguaí	6	22	23	31.1	32.6	<1	<1	83.3	<1	1.7×10^2	83.3	<10	7.7×10^3
Rio Grande	6	20	21	0	0.1	<1	1.3×10^3	100	5.8×10^1	2.9×10^2	100	3.2×10^3	$1.5 \times 10^6 \psi$

The symbol ψ refers to estimated values.

consequent loss of ambient chitin substrates for attachment, particularly in the case of chitinolitic bacteria such as vibrios. Dead zooplankton will eventually sink and accumulate in bottom sediments, and because zooplankton carcasses provide protection to bacteria from environmental stresses (Tang et al., 2010) it is likely that plankton-associated vibrios have been underestimated in BW tanks. A similar "protective refugia" may be found in biofilm matrices, which can sequester free-living bacteria during multiple fill and discharge cycles (Drake et al., 2005, 2007).

The same explanation may apply for the low detection rates observed for both associated and free-living *V. cholerae* O1 and non-O1 in BW samples. *V. cholerae* O1 often appears in the so-called viable but non-culturable (VBNC) form, requiring immunological and molecular tests for detection (Colwell and

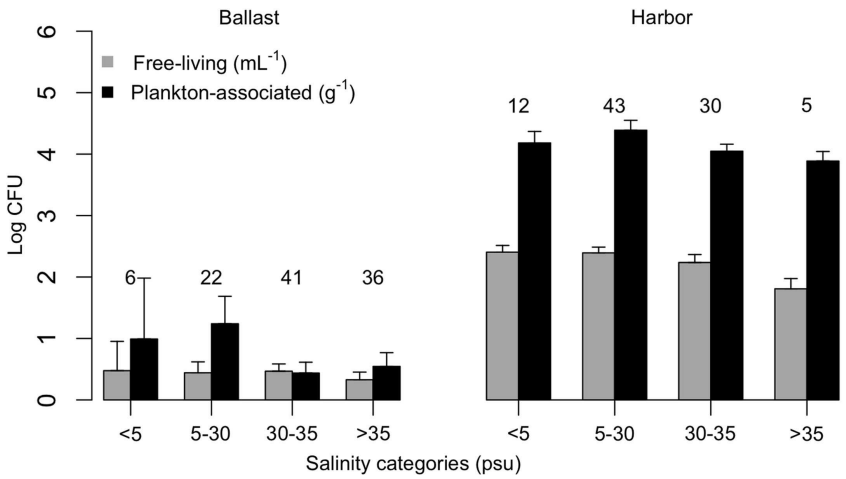

FIGURE 2 | Free-living (CFU mL^{-1}) and plankton-associated (CFU g^{-1}) vibrio concentration in ballast water (left panel) and harbor (right panel) samples, according to salinity categories. Number of samples analyzed are indicated on top of standard error bars. CFU, colony-forming units.

Table 3 | Characterization of free-living and plankton-associated *Vibrio cholerae* strains isolated from environmental samples collected in Brazilian harbor areas.

Harbor areas	*V. cholerae*		
	Free-living (F) or plankton-associated (A)	**Serogroup**	**Genotype**
Belém	F	O1	*ctx*A$^-$/*tcp*A$^-$
		Non-O1	*ctx*A$^+$/*tcp*A$^+$
Recife	F	O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$
		Non-O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$, *ctx*A$^-$/*tcp*A$^+$
	A	Non-O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$, *ctx*A$^-$/*tcp*A$^+$
Itaguaí	F	Non-O1	*ctx*A$^-$/*tcp*A$^-$
Santos	F	O1	*ctx*A$^+$/*tcp*A$^-$
		Non-O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$
	A	O1	*ctx*A$^+$/*tcp*A$^+$
		Non-O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$
Paranaguá	F	O1	*ctx*A$^+$/*tcp*A$^+$
		Non-O1	*ctx*A$^+$/*tcp*A$^+$, *ctx*A$^+$/*tcp*A$^-$
	A	Non-O1	*ctx*A$^-$/*tcp*A$^-$

Genotypes ctxA and tcpA are genes codifying for cholera toxin and toxin co-regulated pilus, respectively.

Huq, 1994; Huq et al., 2000). For instance, the use of direct immunofluorescence microscopy has yielded high detection rates for *V. cholerae* O1 in estuarine and coastal sites in Brazil (67–90%, Martins et al., 1993; Martinelli Filho et al., 2010) and in ship's BW in the United States (Ruiz et al., 2000).

Vibrio assessment in harbor and coastal sites is critical to evaluate the microbiological risk of BW discharges and to provide a baseline for BW management and surveillance programs. Cholera and toxigenic *V. cholerae* O1 offer a useful model to examine the transportation of pathogenic microorganisms by BW (Ruiz et al., 2000). This association has essentially emerged from the recognition that BW played a role in the spread of the seventh cholera pandemic in South America in the early 1990s (Colwell, 1996). The environmental constraints in cholera epidemics have also been emphasized by Colwell (1996) who suggested that peaks in vibrio abundance were linked to the increase in copepod production during El Nino event. Additionally, Baker-Austin et al. (2012) reported the association of warming patterns to emergence of *Vibrio* infections in the Baltic area.

Contrasts in the aquatic ecosystem between donor and receiving regions are important in defining the likelihood of a successful invasion, including that of an emerging disease. Our finding of a toxigenic *V. cholerae* O1 strain (*ctx*A$^+$, *tcp*A$^+$) in BW from a ship that had just arrived in the Belém harbor yields a good example: the toxigenic bacteria thrived in a much more saline environment within the tank (salinity of 29.8 psu) compared to the surrounding environment, as Belém is a freshwater harbor. This most likely helped to prevent the arriving toxigenic strain from establishing itself in that location.

Nevertheless, the presence of toxigenic *V. cholerae* O1 in BW tanks from ships arriving in Brazilian ports, as well as in local harbor areas, is a matter of concern. We detected toxigenic (*ctx*A$^+$, *tcp*A$^+$) *V. cholerae* non-O1 strains, which potentially interact with naturally occurring non-toxigenic serogroup O1 or may convert itself to *V. cholerae* O1 by conjugation, or seroconversion (Chiang and Mekalanos, 1999). The frequency of environmental strains of non-O1 *V. cholerae* containing virulence-associated factors is low (Rivera et al., 2001; Vital Brazil et al., 2002), however the emergence of *V. cholerae* O1 toxigenic strains (*ctx*A$^+$) resulting from lysogenic infection and conversion by the filamentous phage CTXϕ (Waldor and Mekalanos, 1996) and other genetic virulence elements has been described (Karaolis and Kaper, 1999). Once introduced in the environment by contaminated BW discharge,

Table 4 | *Vibrio* frequency and concentration range on coastal areas of São Paulo State, Brazil.

Coastal areas	Free-living vibrios (CFU mL^{-1})				Plankton-associated vibrios (CFU g^{-1})			
	n	%	Max	Min	*n*	%	Max	Min
Santos	15	100	1.6×10^3	8.9×10^1	15	100	1.8×10^6	90
São Sebastião Channel	32	100	4.6×10^2	2	32	100	1.4×10^6	6.0×10^2
Ubatuba	8	100	1.3×10^2	2	8	100	8.0×10^5	6.8×10^3

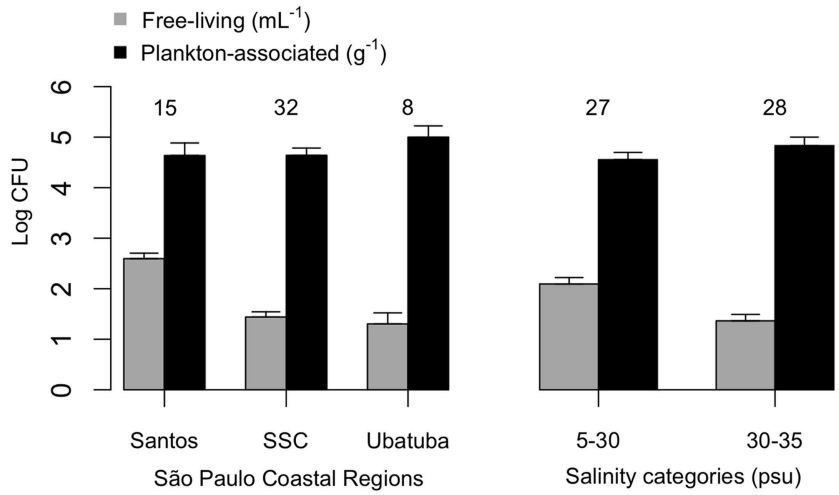

FIGURE 3 | Free-living (CFU mL^{-1}) and plankton-associated (CFU g^{-1}) vibrio concentration from São Paulo coastal areas. Data shown by coastal site (left panel) and salinity categories (right panel). Number of samples analyzed are indicated on top of standard error bars. CFU, colony-forming units.

a toxigenic population of *V. cholerae* O1 may genetically interact with a non-toxigenic native population setting conditions for a cholera outbreak, especially where sanitary conditions are poor. An invasive *V. cholerae* strain could then be further transported by ships to other regions or dispersed naturally by aquatic currents, thereby affecting a large geographic region. The role of coastal eutrophication on vibrio distribution was depicted in our study by the high vibrio abundances found in the Santos area, where poor water management practices prevail.

The use of *V. cholerae* as a model pathogenic bacterium in BW analysis is a crucial approach to ensure the sanitary quality of coastal waters and to prevent the spread of cholera epidemics by maritime transport. Long-term BW surveillance programs will certainly help to minimize the introduction of toxigenic *V. cholerae* into new areas. For that purpose, methods to differentiate pathogenic from non-pathogenic *V. cholerae* populations in aquatic ecosystems have been proposed and successfully implemented (Rivera et al., 1995, 2003), and recent achievements in microbial detection using lab-on-a-chip approaches (e.g., Jung et al., 2011) may prove extremely useful for BW monitoring applications.

CONCLUSION

Despite research and management initiatives carried out by a number of organizations worldwide, shipping continues to represent a threat as a major vector for the transfer of invasive aquatic species. A considerable effort has been given to the study and control of non-indigenous plants, algae, and invertebrates transported by cargo ships, but fewer investigations exist on the role of BW discharge in the spread of bacteria across biogeographical provinces and in the dissemination of emerging aquatic diseases. We believe these studies are essential, and strongly encourage the engagement of microbiologists, plankton ecologists, and engineers in the search for novel solutions for BW monitoring systems. Future developments in fast and reliable detection techniques are essential to implement cost-effective and environmentally sound BW management programs.

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