ROLES AND MECHANISMS OF PARASITISM IN AQUATIC MICROBIAL COMMUNITIES

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ROLES AND MECHANISMS OF PARASITISM IN AQUATIC MICROBIAL COMMUNITIES

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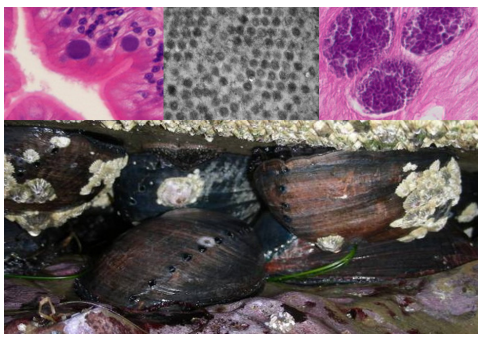

Withering syndrome in black abalone. Top left: Withering syndrome rickettsia-like organisms (WS-RLO); Top middle: Transmission electron micrograph of phage hyperparasites within a WS-RLO; Top right: WS-RLO infected with phage. Bottom image: Black abalone from a field site in central California.
Images by Carolyn Friedman, School of Aquatic and Fishery Sciences, University of Washington

Next Generation Sequencing technologies are increasingly revealing that microbial taxa likely to be parasites or symbionts are probably much more prevalent and diverse than previously thought. Every well studied free-living species has parasites; parasites themselves can be parasitized. As a rule of thumb, there is an estimated 4 parasitic species for any given host, and the better a host is studied the more parasites are known to infect it. Therefore, parasites and other symbionts should represent a very large number of species and may far outnumber those with 'free-living' lifestyles. Paradoxically, free-living hosts, which form the bulk of our knowledge of biology, may be a minority! 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 the environment, comprising viruses, prokaryotes and eukaryotes. This Frontiers Research Topic sought to provide a broad overview but concise, comprehensive, well referenced and up-to-date state of the art for everyone involved with microbial parasites in aquatic microbial ecology.

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EDITORIAL
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Editorial: Roles and mechanisms of parasitism in aquatic microbial communities

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Our research topic on the roles and mechanisms of parasitism in aquatic microbial communities should be of broad interest, given that there are probably more parasitic species than free-living ones (Windsor, 1998), a hypothesis increasingly supported by next generation sequencing technologies of microbial taxa (Sime-Ngando and Niquil, 2011). We know little about the parasites of microbes, but recent research suggests that they affect food-web dynamics, biogeochemical cycling, the functioning of ecosystems and related services, and host evolution. Furthermore, several new research topics, such as interactomics, molecular dialogue, host manipulation by parasites (Cézilly et al., 2014; Biron et al., 2015), "beneficial" parasites (Roossinck, 2011; Parfrey et al., 2014), priming of the host immune system (Llewellyn et al., 2014), microbiomics (Llewellyn et al., 2014), and tripartite symbiosis (Rohwer and Thurber, 2009; Gleason et al., 2014), are filled with interesting questions. We ordered the thirteen papers in this issue according to the biological complexity of parasites, from phages-bacteria, phage-bacteria-animal, microparasite-microbe, microparasite-microhyperparasite-microbe, microparasite-animal to macroparasite-microbe interactions.

New findings in the papers include the conceptualization of viral lifestyles and the extension of their role as microbe killers, cell partners, or metabolic manipulators (Sime-Ngando, 2014). These relationships have applications for economics and conservation. For instance, shore-based abalone aquaculture can discharge pathogens like the intracellular bacterium *Candidatus Xenohaliotis californiensis* (WS-RLO), with potential impacts to wild abalone (Lafferty and Ben-Horin, 2014). However, a novel bacteriophage now infects the WS-RLO, improving the survival of infected abalone and thereby offering a potential tool for population management via phage therapy (Friedman et al., 2014). The importance of such tripartite interactions relates to Gleason et al. (2014) argument that parasites of parasites may increase the complexity of food webs, and play significant roles in suppressing diseases of animals, plants, or algae. The ecological importance of such disease dynamics is illustrated well by new quantitative data and modeling that shows how during blooms of inedible algae in freshwater lakes, (i) chytrid parasites of phytoplankton are able to shape aquatic ecosystems by altering sinking fluxes or determining system stability (Kagami et al., 2014), and (ii) divert about 20% of primary production to edible zoospores that comprise 50–57% of the zooplankton diet (Rasconi et al., 2014). This work is remarkable given how challenging it is to diagnose parasites of microbes in natural systems (Karpov et al., 2014). Economic incentive for improved diagnosis stems from the effects of infection dynamics on commercial-scale algal monocultures for bioenergy and chemical production (Carney and Lane, 2014). Several topics in this collection deal with microbial parasites and the microbiome of fishes and animals, demonstrating, for example, that viral, prokaryotic and small-eukaryotic parasites affect conservation and food security (Gozlan et al., 2014). For instance, indigenous

microbiota affects innate and adaptive immunity, fish digestion, and nutrient metabolism (Llewellyn et al., 2014). The extent to which aquatic microbes differ from other small eukaryote communities is highlighted by Parfrey et al. (2014) who use high-throughput sequencing to consider how microbes in the mammalian gut reflect both host phylogeny and diet, and are distinctive from those in aquatic and terrestrial habitats. The microbiome might even influence host behavior as a result of the molecular crosstalk between a manipulative parasite and its host, disturbing the synthesis of neuroactive molecules (Biron et al., 2015). We end with Cézilly et al. (2014), who consider the hypothesis of conflict vs. cooperation in host manipulation, and provide empirical evidence that microorganisms can have synergistic and antagonistic interactions with co-occurring parasites.

We hope the contributions to this collection bring a new focus to the aquatic sciences. Microbial interactions are clearly important and largely unknown. There are still methodological barriers to assessing prokaryotic and eukaryotic parasites of aquatic microbes (Sime-Ngando and Niquil, 2011), although recent advancements provide new opportunities (Marano et al., 2012), which we expect will lead to, a predictive understanding of the role of parasitism in aquatic systems in particular, and of aquatic ecosystem functioning in general.

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Environmental bacteriophages: viruses of microbes in aquatic ecosystems

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Since the discovery 2–3 decades ago that viruses of microbes are abundant in marine ecosystems, viral ecology has grown increasingly to reach the status of a full scientific discipline in environmental sciences. A dedicated ISVM society, the International Society for Viruses of Microorganisms, (http://www.isvm.org/) was recently launched. Increasing studies in viral ecology are sources of novel knowledge related to the biodiversity of living things, the functioning of ecosystems, and the evolution of the cellular world. This is because viruses are perhaps the most diverse, abundant, and ubiquitous biological entities in the biosphere, although local environmental conditions enrich for certain viral types through selective pressure. They exhibit various lifestyles that intimately depend on the deep-cellular mechanisms, and are ultimately replicated by members of all three domains of cellular life (Bacteria, Eukarya, Archaea), as well as by giant viruses of some eukaryotic cells. This establishes viral parasites as microbial killers but also as cell partners or metabolic manipulators in microbial ecology. The present chapter sought to review the literature on the diversity and functional roles of viruses of microbes in environmental microbiology, focusing primarily on prokaryotic viruses (i.e., phages) in aquatic ecosystems, which form the bulk of our knowledge in modern environmental viral ecology.

Keywords: aquatic ecosystems, viruses, lysis, lysogeny, bacteria, horizontal gene transfers, food web dynamics, biogeochemical cycling

INTRODUCTION

With the discovery few decades ago that viral parasites of microbes are abundant in marine ecosystems (Torrella and Morita, 1979; Bergh et al., 1989), aquatic viral ecology has increasingly grew to reach the status of full scientific discipline in environmental sciences, with the recent launch of a dedicated ISVM society, i.e., the International Society for Viruses of Microorganisms[1]. As infectious agents of potentially all types of living cells, viruses are the most abundant biological entities in the biosphere. They are ubiquitous components of the microbial food web dynamics in a great variety of environments, including the most extreme ecosystems. Moreover, in spite of the difficulties to routinely observe and describe biological nanoparticles, combined with the absence of conserved evolution tracers such as RNA ribosomal genes, we now consider that viruses represent the greatest reservoir of non-characterized genetic diversity and resources on the earth (Suttle, 2007). They contain genes that code for essential biological functions such as photosynthesis (Lindell et al., 2005), making their hosts powerful vehicles for genetic exchanges in the environment. Because lytic viruses killed their hosts, they play fundamental roles in cycling nutrients and organic matter, structuring microbial food webs, governing microbial diversity and, to a lesser extent, by being a potential food source for protists (Sime-Ngando and Colombet, 2009). As symbionts, viruses can also form long-lived association with their specific hosts, reducing their fitness, or allowing infected hosts to remain strong competitors through mutualistic symbioses (Roossinck, 2011). In addition, the discovery and characterization of the unique group of archaeal viruses are influencing the field of prokaryotic virology, increasing our knowledge on viral diversity and changing perspectives on early stages of evolution (cf. Prangishvili et al., 2006).

Recent studies in aquatic viral ecology are source of novel knowledge related to the biodiversity of living things, the functioning of ecosystems, and the evolution of the cellular world. Viruses exhibit various life strategies that intimately depend on the deep-cellular mechanisms, and are ultimately replicated by all members of the three domains of cellular life: Bacteria, Eukarya, and Archaea. This establishes viruses as microbial killers (i.e., bad viruses) but also as cell partners and manipulators (i.e., good viruses) in the world of aquatic ecosystem (Rohwer and Thurber, 2009; Roossinck, 2011). The present chapter sought to review the literature on the diversity and functional roles of viruses in aquatic microbiology, focusing on prokaryotic viruses (i.e., bacteriophages) which form the bulk of our knowledge in aquatic viral ecology.

DEFINITION AND LIFESTYLES

Viruses are biological entities consisting of single- or double-stranded DNA or RNA surrounded by a protein and, for some of them, a lipid coat. In aquatic systems, most viruses are tailed or untailed phages, with a capsid diameter often smaller than 250 nm, based on direct transmission electron microscopy observation. Viruses have no intrinsic metabolism and need

[1]http://www.isvm.org/

the intracellular machinery of a living and sensitive host cell for all processes requiring energy. They have various life cycles, all starting with diffusive passive fixation on specific receptors (often transporter proteins) present at the surface of a host cell, followed by injection of the viral genome into the host cell. In the lytic cycle, the viral genome induces the synthesis of viral constituents, including the replication of the viral genetic material. A number of progeny viruses are then produced and released into the environment by the fatal rupture of the host cell.

In the chronic cycle, the progeny viruses are episodically or constantly released from the host cell by budding or extrusion, without immediate lethal events. This cycle is less well known in aquatic microbiology, but it is common in metazoan viruses such as Herpes and Hepatitis viruses or rhabdoviruses. Chronic viral infection is a dynamic and metastable equilibrium process which ends with the lysis of the host cell after serial budding of lipid membrane-coated viruses, as seen in hosts of the marine protist *Emiliania huxleyi* (Mackinder et al., 2009). Recently, chronic infection without host lysis has been reported for the first time in the marine primary producer *Ostreococcus tauri*, where the low rate of viral release through budding ($1–3$ viruses cell^{-1} day^{-1}) allows cell recovery and the stable coexistence of viruses and their hosts (Thomas et al., 2011; Clerissi et al., 2012).

In the lysogenic cycle, the viral genome integrates the genome of the host cell and reproduces as a provirus (or prophage) until an environmental stress to the immune host cell sets off a switch to a lytic cycle. Both the provirus and the host cell benefit from lysogeny. Lysogeny provides a means of persistence for viruses when the abundance of the host cells is very low. Prophages may affect the metabolic properties of host cells which can acquire immunity to superinfections and new phenotypic characteristics such as antibiotic resistance, antigenic changes, and virulence factors, resulting in niche expansion for viral hosts (**Figure 1**). A variant to the lysogenic cycle is the so-called carrier state or pseudolysogenic cycle, where the viral genome is not integrated with the host genome but rather remains in an "inactive state" within the host cell. There is no replication of the viral genome which is segregated unequally into progeny cells, most likely for a few generations. Pseudolysogenic viruses probably occur in very poor nutrient conditions where host cells are undergoing starvation and cannot offer the energy necessary for viral gene expression.

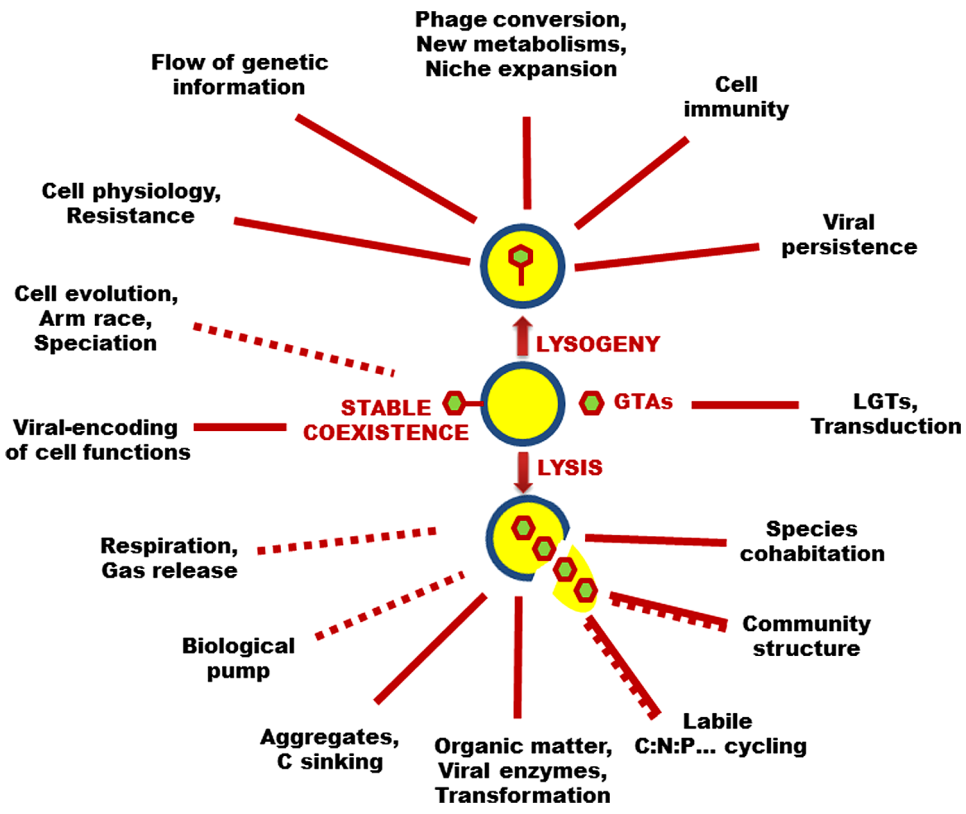

FIGURE 1 | Virus–microbe interactions range in a gradient from true non-lethal parasitism (i.e., stable coexistence) to fatal lytic infection (lysis), with intermediate mutualistic lifestyles (lysogeny and pseudolysogeny). Because of the existence of such a large panel of lifestyles and in conjunction with the fact that all types of cells are sensitive to unique viruses, these biological entities are considered the most diverse, abundant, and ubiquitous biological entities in the biosphere where they have tremendous effects on the diversity of living things, the functioning of microbial ecosystems, and the evolution of the cellular world. Some of these direct (solid lines) and indirect (dashed lines) effects on aquatic microbial processes are highlighted in this figure. Please refer to the main text for abbreviations.

TAXONOMY

There are three domains of life – Archaea, Bacteria, and Eukarya – that consist only of cellular organisms (Woese et al., 1990). Because viruses lack the ribosomal RNA nucleotide sequence upon which these cellular domains of life are based, they cannot be integrated into the cellular tree of life (Breitbart et al., 2002; Rohwer and Edwards, 2002), although susceptibility to virus infection is a common feature of all members of the three domains of life. In the absence of universal evolution markers for the entire viral world, viruses have been grouped by many different methods, according to various criteria: the nature of the host, the characteristics of the free virions (phenotype, genotype, resistance to organic solvents for viruses with lipid coat, etc.), or even the name of the related illness, the laboratory or the researcher working on the targeted viruses. The 9th report by the International Committee on Taxonomy of viruses (ICTV)[2] includes 6 orders, 87 families, 19 subfamilies, 349 genera, and 2284 virus and viroid species, defined as a group of viruses that constitutes a replicative lineage and occupies a particular ecological niche (Regenmortel, 1992). Hurst (2011) introduced the idea that the taxonomy of viruses and their relatives could be extended to the domain level, and suggests the creation of an additional biological domain that would represent the acellular infectious agents that possess nucleic acid genomes or the genomic acellular agents. The proposed domain title is Akamara, whose derivation from the Greek (a + kamara) would translate as "without chamber" or "without void." The domain is divided into two kingdoms. The kingdom Eurivia includes true viruses and those satellite viruses whose genomes code for their own capsid proteins, and is separated in two Phyla (RNA and DNA viruses). The second kingdom (Viroidia) forms one phylum that includes viroids along with other groups of related agents whose genomes likewise do not code for their structural "shell" proteins.

A new way to classify phages has also been proposed based on the complete sequences of 105 viral genomes. This so-called phage proteomic tree places phages relative to their neighbors and all other phages included in the analysis, which is a method that can be used to predict aspects of phage biology and evolutionary relationships, and to highlight genetic markers for diversity studies (Rohwer and Edwards, 2002). The approach is useful for those phages whose complete genomes have been deciphered (i.e., a minority of environmental viruses); primarily those belonging to a common pool of genes (e.g., following genetic recombination), or which have evolved from a common ancestor. It is well known that the genomes of some phages, if not most, are mosaics of genes from various sources, including other phages and their hosts (Hendrix et al., 1999).

PHENOTYPIC TRAITS

The first descriptions of the global diversity of viruses are from the general forms of virus-like particles observed via transmission electron microscopy. In aquatic samples, viral phenotypes are limited, mainly including tailed or untailed particles with capsid heads, characteristics of bacteriophages. Tailed phages belong to the order Caudovirales, all of which are double-stranded DNA viruses that generally represent 10–40% of the total abundance of viruses in aquatic systems (see comparative tables in Wommack and Colwell, 2000; Sime-Ngando and Colombet, 2009). Within Caudovirales, three families emerge as quantitatively dominant: Siphoviridae with long, non-contractile tails (e.g., Phage lambda), Podoviridae with a short, non-contractile tail (e.g., Phage T7), and Myoviridae with contractile tails of variable length (e.g., Phage T4). In most studies, non-tailed capsids dominate viral abundances. This may be an artifact due to the effects of mechanic shocks resulting from handling, primarily ultracentrifugation (Colombet et al., 2007), because 96% of the 5500 specimens of described bacteriophages are tailed particles (Ackermann, 2007). However, a recent global morphological analysis of marine viruses suggested that non-tailed viruses, which comprised 50–90% of the viral particles observed, might represent the most ecologically important component in natural viral communities (Brum et al., 2013).

Phenotypic traits and viral morphs in aquatic viruses are cryptic of the selective pressures faced by these communities, and provide insight into host range, viral replication and function (Suttle, 2005). For instance, myoviruses are mostly lytic with a large spectrum of sensitive hosts, which is a competitive advantage that can be assimilated to r-strategist species thriving with high proliferation rates in fluctuating environments. In contrast, podoviruses are more highly specific to their hosts, with siphoviruses being intermediate between myo- and podoviruses. In addition, several siphoviruses can encode their genome into their hosts for several generations (i.e., lysogeny), which can be rather assimilated to K-strategist species, characteristics of stable environments. Combined with the capacity of viruses to potentially face almost all types of environments and the related interfaces (Hurst, 2011), the ability of viruses to develop along the r-K-selection continuum, i.e., from typical r (e.g., prokaryotes) to K (e.g., vertebrates) strategists (Suttle, 2007), may help to explain their ubiquity, hence the notion of the virosphere (i.e., viral biosphere).

GENOMIC DIVERSITY

ICTV-reported viral species are mostly known from their isolated hosts in laboratory cultures which, in the case of environmental samples, may not exceed 1% of the total prokaryotes (Hugenholtz et al., 1998). This implies that the diversity of environmental viruses is huge, although the bulk of the estimated 10^{31} viruses in the biosphere is unknown (Rohwer and Edwards, 2002). In addition to whole genome sequencing of specific phages (Allen et al., 2011) or genomes assembled from metagenomics datasets (Rosario et al., 2009), molecular approaches applied to uncultured complex communities are thus critical and offer windows to the largest uncharacterized reservoir of diversity on the earth (Hambly and Suttle, 2005). Polymerase chain reaction (PCR)-based methods are restricted to chosen viral groups as no gene is universally conserved among viruses, while part of the existing diversity of these viral groups is missed because PCR primers are based on previously identified sequences described in public databases. Viral metagenomics gives access to an exhaustive view of uncultured viral diversity (Breitbart et al., 2002), and has so far revealed an

[2]http://www.ictvonline.org/index.asp?bhcp=1

important unknown diversity and an unexpected richness of viral communities (Edwards and Rohwer, 2005).

Despite the ecological importance of viruses, a fundamental hindrance to their integration into microbial ecology studies is the lack of suitable reference bacteriophage genomes in reference databases. Currently, only eight bacterial phyla (Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Cyanobacteria, Chlamydiae, Tenericutes, and Deinococcus-Thermus) of 29 phyla with cultured isolates have sequenced phage representatives, contributing only 0.001% of genome sequence databases (Bibby, 2014). From these few phage genomes, comparative genomics have revealed an impressive level of genomic diversity and novelties as well as hypotheses on potential adaptation of phage genome to aquatic environments. For example, comparison of 26 T4-like genomes of myoviruses infecting diverse marine cyanobacteria (*Prochlorococcus* or *Synechococcus*) has revealed highly syntenic hierarchical cores of genes, with DNA replication genes observed in all genomes, followed by previously described, virion-structural and various hypothetical genes. Beyond previously described cyanophage-encoded photosynthetic and phosphate stress genes, genes involved in various putative functions (e.g., phytanoyl-CoA dioxygenase, 2-oxoglutarate) were indeed described, as well as non-core genes that may drive complex niche diversification (Sullivan et al., 2010). The unveiling of the first genome of a deep-photic marine cyanobacterial siphovirus highlighted the prevalence of lysogenic lifestyle and significant divergence and size differences with previously sequenced siphoviruses, and the absence of photosynthetic genes which have consistently been found in other marine cyanophages (Sullivan et al., 2009). Similarly, the genomic and functional analysis of a novel marine siphovirus of marine *Vibrio* revealed a larger genome (80,598 bp) compared to that of most known siphoviruses, with a novel shell symmetry that confers a remarkable stability to a variety of physical, chemical, and environmental factors (Baudoux et al., 2012). Whole genome sequencing/reconstruction of phages that are currently unrepresented in the database will thus likely provides deep insights into and have a significant impact on our view of viral diversity, ecology, and evolution, while providing molecular tools for the study of groups of viruses.

Whole genome comparisons indeed have also shown that there are conserved genes shared among all members within certain viral taxonomic groups. These conserved genes can be targeted using PCR amplification and sequencing for diversity studies of groups of cultured and environmental viruses. Examples of such genes are structural proteins such as gp20, which codes for the capsid formation in T4 phage-like viruses, DNA polymerases for T7-like podophages, or the RNA-dependent RNA polymerase fragment, which has been used to identify novel groups of marine picornaviruses (Culley et al., 2003). All of the conserved gene studies suggest that environmental viral diversity is high and essentially uncharacterized (Breitbart and Rohwer, 2005).

For the whole environmental communities, molecular fingerprinting approaches that separate PCR-generated DNA products, such as denaturing gradient gel electrophoresis (DGGE, Short and Suttle, 2002) and pulse-field gel electrophoresis (PFGE, Wommack et al., 1999), have been widely used but with limited results, restricted to double-stranded DNA viruses. With this approach, the genome size of aquatic viruses fluctuates from 10 to about 900 kb, with mean ranges of 10–630 kb and 10–660 kb in marine and freshwater systems, respectively. The frequencies of the distribution of genome size classes are multimodal, with peaks in the interval < 70 kb and a mean at 50 kb.

An introduced fingerprinting approach adapted to viruses, randomly amplified polymorphic DNA-PCR (RAPD-PCR), allows sampling of viruses at the genetic level without requiring viral isolation or previous sequence knowledge (Winget and Wommack, 2008). RAPD-PCR is accurate in assessing DNA viral richness in water samples by using single 10-mer oligonucleotide primers to produce amplicons (PCR-generated DNA fragments) and banding patterns, with each likely representing a single amplicon that originates from viral template DNA. Such an approach has been demonstrated to match observations from other community profiling techniques, revealing more temporal than spatial variability in viroplankton assemblages. Hybridization probes and sequence information can also be easily generated from single RAPD-PCR products or whole reactions, providing a tool for routine use in high-resolution viral diversity studies by providing assemblage comparisons through fingerprinting, probing, or sequence information.

Metagenomics has revolutionized microbiology by paving the way for a culture-independent assessment and exploitation of microbial and viral communities present in complex environments (Simon and Daniel, 2011). Metagenomic viral analyses or virome studies suggest that environmental viral diversity is high and essentially uncharacterized (Angly et al., 2006; Roux et al., 2012). Metagenomic analyses of 184 viral assemblages collected over a decade and representing 68 sites in four major oceanic regions showed that most of the viral DNA and protein sequences were not similar to those in the current databases (Angly et al., 2006). Global diversity was very high, presumably several hundred thousand species, and regional richness varied on a North–South latitudinal gradient. However, most viral species were found to be widespread, supporting the idea that marine viruses are widely dispersed and that local environmental conditions enrich for certain viral types through selective pressure. A study on comparative viral metagenomics highlighted that freshwater, marine, and hypersaline environments were separated from each other despite the vast geographical distances between sample locations within each of these biomes, suggesting a genetic similarity between viral communities of related environments (Roux et al., 2012).

Interrogation of microbial metagenomic sequence data collected as part of the Sorcerer II Global Ocean Expedition (GOS) also revealed a high abundance of viral sequences, representing approximately 3% of the total predicted proteins in the 0.1–0.8 μm size fraction of the plankton (Williamson et al., 2008). Viral sequences revealed hundreds to thousands of viral genes, encoding various metabolic and cellular but mostly structural functions. Quantitative analyses of viral genes of host origin confirmed the viral nature of these sequences and suggested that significant portions of aquatic viral communities behave as reservoirs of such genetic material. Distributional and phylogenetic analyses of these host-derived viral sequences also suggested that viral acquisition

of environmentally relevant genes of host origin is a more abundant and widespread phenomenon than previously appreciated. The predominant viral sequences identified within microbial fractions originated from tailed bacteriophages and exhibited varying global distributions according to viral family. The recruitment of GOS viral sequence fragments against 27 complete aquatic viral genomes revealed that only one reference bacteriophage genome was highly abundant and was closely related, but not identical, to the cyanobacterial myovirus P-SSM4 of *Prochlorococcus* hosts, suggesting that this virus may influence the abundance, distribution, and diversity of one of the most dominant components of small phytoplankton in oligotrophic oceans (Williamson et al., 2008).

Overall, metagenomic analysis of viruses increasingly suggests novel patterns of evolution, changes the existing ideas on the composition of the virus world, and reveals novel groups of viruses and virus-like agents (Kristensen et al., 2010). The gene composition of marine DNA viromes is dramatically different from that of known bacteriophages. The virome is dominated by unknown genes, many of which might be contained within virus-like entities such as gene transfer agents (GTA), which are host DNA carrier particles (Lang et al., 2012). Analysis of marine metagenomes thought to consist mostly of bacterial genes revealed a variety of sequences homologous to conserved genes of eukaryotic nucleocytoplasmic large DNA viruses, resulting in the discovery of diverse members of previously undersampled groups and suggesting the existence of new classes of virus-like agents.

Unexpectedly, metagenomics of marine RNA viruses showed that representatives of one superfamily of eukaryotic viruses, the picorna-like viruses, dominate the RNA virome (Kristensen et al., 2010). Marine RNA viruses are almost exclusively composed of those that infect eukaryotes (Lang et al., 2009), primarily protists (Culley et al., 2007). This was confirmed in a recent quantitative study where the comparison of the total mass of RNA and DNA in viral fraction suggests that the abundance of RNA viruses equaled or exceeded that of DNA viruses in coastal seawater (Steward et al., 2013). Similar findings were also reported in freshwater systems (Roux et al., 2012). Because picorna-like viruses have small genomes, they are at or below the detection limit of common fluorescence-based counting methods, implying that protists contribute more to marine viral dynamics than one might expect based on their relatively low abundance. Similarly, a recent metagenomic study from temperate and subtropical seawater has highlighted 129 genetically novel and distinct viruses based on complete genome assemblages, all of which were single-stranded DNA viruses mostly known as economically important pathogens of plants and animals (Labonté and Suttle, 2013). The discovery of RNA and ssDNA viruses is a significant departure from the prevailing view of aquatic viruses which are assumed to mostly contain double-stranded DNA and infect bacteria. It is thus likely that we are missing a significant fraction of viruses in aquatic ecosystems, which probably is one of the many reasons that may help explain the apparent discrepancy between genome-derived and metagenome-derived diversity of viruses. These reasons are highlighted in Ignacio-Espinoza et al. (2013).

ABUNDANCE, DISTRIBUTION, AND BIOGEOGRAPHY

Viruses were first suspected as abundant particles in the sea in the late 1970s (Torrella and Morita, 1979), which was confirmed one decade later with the discovery that 1 ml of sea water contains millions of viruses (Bergh et al., 1989). First estimates were variable and inaccurate because they were based on manipulated (i.e., ultracentrifuged) samples observed at high magnification using transmission electron microscopy. More accurate and reproducible estimates were provided later using direct epifluorescent microscopy or flow cytometry, yielding viral abundances that exceed those of Bacteria and Archaea by an overall average of about 15-fold (Bettarel et al., 2000). It is important to use fluorochrome dyes (e.g., SYBR Gold) that bind to ssDNA and ARN viruses which have been proved, based on metagenomics, to be much more abundant in natural aquatic systems than expected (Lang et al., 2009; Holmfeldt et al., 2012). Furthermore, in this context of methodological difficulties for accurate estimates of the numerical abundance of natural viruses, it is important to stress the recent discovery that DNA associated with membrane-derived vesicles, gene transfer agents, or cell debris can produce fluorescent dots that can be confused with viruses (Forterre et al., 2013). This targets a critical problem that needs to be bypassed in the future because many bacterial species, including the dominant marine cyanobacterium *Prochlorococcus* spp., release extracellular vesicles which have roles in various processes (e.g., quorum sensing, virulence, horizontal gene transfers, etc.), and were recently demonstrated to have a significant effect on carbon cycling in marine ecosystems (Biller et al., 2014).

Viral abundance generally increases with the increasing productivity of aquatic ecosystems and, as a consequence, decreases from freshwater to marine ecosystems, from costal to oceanic zones, and from the surface to the bottom of the euphotic layer (Sime-Ngando and Colombet, 2009). The abundance of viruses in individual aquatic systems appears to be independent of salinity but related to the biomass of primary and secondary producers, as well as to seasonal effects (Wilhelm and Matteson, 2008). In the dark ocean (i.e., meso- and bathypelagic zones), where about 75% of prokaryotic biomass and ca. 50% of prokaryotic carbon production in the world ocean occur (Aristegui et al., 2009), high abundance of viruses was observed (Parada et al., 2007). Similarly, two deep marine sediment studies from Ocean Drilling Project samplings in Saanich Inlet, Canada (Bird et al., 2001), and on the Eastern margin of the Porcubine Seabight (Middelboe et al., 2011) have reported abundant viruses and prokaryotes in >100 m sediment cores aged from 0 to 14,000 years and from 0.5 to 2 million years, respectively. On a volumetric basis, viral abundances in sediments exceed 10–1000 times that in the water column, representing active and mostly endemic components of benthic environments (Danovaro et al., 2008), although visibly infected cells are often scarce (Filippini et al., 2006). Because the relative abundances of Archaea increase in the dark deep ocean and freshwater lakes, viruses of Archaea are also expected to be abundant there, as recently suggested by highly complex, diverse morphologies observed in a deep-dark permanently anoxic freshwater lake, some of which being putatively new for science (Borrel et al., 2012). Thus, it is likely that the ecology of the deepest and benthic

waters where eukaryotes are constrained by poor oxygen conditions is essentially driven by the dark viral loop (dissolved organic matter–prokaryotes–viruses) processes (Colombet and Sime-Ngando, 2012).

According to time, viral abundances fluctuate on diverse scales, from minutes to years, often in association with prokaryotes, which offer the major reservoir for hosts (Wommack and Colwell, 2000). Surprisingly, there is only one to two orders of magnitude variation in virus abundance among systems (c. 100-fold; 10^9–10^{11} virus particles l^{-1}) in spite of more than three orders of magnitude variation in the planktonic biomass, as one ranges from either coastal to offshore or from surface to deep-water environments (Wilhelm and Matteson, 2008). This may help to explain why the virus-to-prokaryote ratios (VBRs) fluctuate substantially, with an overall increase from 3 to 10 in oligotrophic marine systems to 6–30 in productive freshwaters where the burst size (i.e., the number of viruses release per lyzed host cell) and the contact and infection rates are generally higher (Sime-Ngando and Colombet, 2009). The higher VBRs in productive lakes may also reflect the increasing relative abundance of non-bacteriophage viruses along the trophic gradient of aquatic systems (Bettarel et al., 2003). Virus abundances in freshwaters appear to vary more strongly on seasonal scales than in marine environments, especially in lakes that undergo pronounced seasonal cycles, although the linkages between seasonal cycles and virus abundance remain unresolved in the absence of long-term studies (Wilhelm and Matteson, 2008). Evidence that viral abundance across oceans and lakes is driven by different factors was provided based on case studies, including bacterial and cyanobacterial abundances, and chlorophyll-*a* concentration as significant variables in lakes, bacterial and cyanobacterial abundances for coastal Pacific Ocean, and bacterial abundance and chlorophyll-*a* concentration for coastal Arctic Ocean (Clasen et al., 2008). However, this mainly concerns free-floating viruses in the water column. Methodological progress based on confocal laser scanning microscopy in combination with lectin and nucleic acid staining has demonstrated that viruses trapped in organic aggregates are much more abundant than in the water column, ranging from 10^8 to 10^{14} viruses l^{-1}. Organic aggregates and inorganic particles appear to play a role of viral scavengers or reservoirs rather than viral factories, and can enhance the growth rates of free-living prokaryotic community. The problems and knowledge gaps in virus–particle interactions, and the related research avenues and implications for water-column ecological processes (e.g., microbial diversity, food web structure, biological pump, biogeochemical cycles, etc.), are provided in an excellent review by Weinbauer et al. (2009).

On a global scale, the forces that shape the biogeography of viruses have received very little attention. It is of interest to search for general patterns of microbial and viral biogeography because general ecological theories, actually known solely from "macroscopic" or visible species (e.g., the positive relationship between diversity and area sampled, or the negative one between local abundance and body size), will offer predictive tools in the context of global change. Microorganisms and their viruses have long been considered as ubiquitous, without a biogeography of any sort. This is because their dispersal is thought to be unlimited due to small size, large absolute abundances and the formation of resistant or dormant stages. The ubiquity tenet for microorganisms is the so-called Baas Becking statement "*everything is everywhere, but the environment selects*" (Fontaneto, 2011). The assumption that viruses are ubiquitous across habitats is currently being evaluated and some phages could be globally distributed, while others could be unique and perhaps endemic to specific habitats (Roux et al., 2012), primarily to extreme environments such as deserts (Prigent et al., 2005; Prestel et al., 2008) or deep-dark permanently anoxic volcanic lake sediments (Borrel et al., 2012). It was also extrapolated from metagenomic data that viral diversity could be high on a local scale but relatively limited globally, and that viruses promote horizontal gene transfers by moving between environments (Breitbart and Rohwer, 2005). Further work is required to fully resolve and confirm the drivers of viral large-scale distribution, in conjunction with the improvement of taxonomy, methods, and sampling effort for both viruses and their hosts.

LYTIC ACTIVITY, MICROBIAL MORTALITY, AND BIOGEOCHEMICAL IMPLICATIONS

The diversity and the abundance of total viruses are not always correlated to the lytic activity. Most free-occurring viruses are considered infectious (Suttle, 2005). It is now well accepted that lytic viruses represent one of the main causes of microbial mortality in aquatic systems (**Figure 1**). Based on the direct observation of infected cells, viral-mediated mortality averages 10–50% of the daily production of heterotrophic prokaryotes and approximately equals the bacterivory from grazers in both fresh and marine waters (Fuhrman and Noble, 1995; Pradeep Ram et al., 2005). These values fluctuate largely (from zero to near 100%) depending mostly on the host availability (density and activity), although physicochemical factors such as solar radiations (Wilhelm et al., 1998), temperature (Pradeep Ram et al., 2005) or anoxia (Colombet et al., 2006) can impact the lytic activity of viruses. The populations of lytic viruses ultimately depend on the availability of specific hosts, and could thus respond to the growth rate of the most active hosts. This pattern has the strong feedback effect of preventing species dominance and enhanced species cohabitation within microbial communities, i.e., the so-called phage kills the winner hypothesis (Thingstad and Lignell, 1997). Viral lytic activity was also demonstrated as uneven and heterogeneous for different prokaryotic phenotypes and/or genotypes, a situation which can shape the host diversity and community structure, and thus exert a strong influence on the processes occurring in the plankton food web dynamics (Pradeep Ram et al., 2010).

Because viruses kill microbial hosts which dominate the biological biomass in pelagic systems, including bacteria, archaea, cyanobacteria, protists, and fungi as major partners, they have an overwhelming effect, both directly and indirectly, on the cycling of the major conservative elements (C, N, P, etc.) upon which the food web dynamic is based (Fuhrman, 1999; Wilhelm and Suttle, 1999). It was estimated that the absolute abundance of oceanic viruses results in about 10^{29} infection events day^{-1}, causing the release of 10^8–10^9 tons of carbon day^{-1} from the living biological pool (Suttle, 2007). By exploding microbial cells, lytic viruses are strong catalyzers of the transformation

of living organisms to detrital and dissolved phases available to non-infected microbes. This biogeochemical reaction increases the retention time of organic matter and its respiration in the water column and weakens the trophic efficiency of the food web, but also provides nutrients (e.g., directly or indirectly from mineralization and photodegradation of dissolved organic matter, DOM) to primary producers (**Figure 1**; Weinbauer, 2004). For example, it has been shown that the iron contains in the viral lysis products could fulfill the metabolic requirements of marine phytoplankton (Poorvin et al., 2004). Primary production in the size fraction 2–200 nm can be depleted by about half due to an increase in viral abundance as low as 20%. A modeling exercise suggested that viral lysis of 50% of bacterial production could increase microbial respiration by 27%, while decreasing the grazing efforts from protists and metazoan zooplankton by 37 and 7%, respectively. When adding 7% of viral-mediated loss of phytoplankton and 3% grazing of viruses by phagotrophic flagellates, bacterial respiration increases to 33% (Fuhrman, 1999).

The effects of lytic viruses thus directly affect DOM concentration but also its composition. For example, Lønborg et al. (2013) recently demonstrated that viral lysate from an axenic culture of *Micromonas pusilla* significantly change the DOM composition by increasing the amounts of transparent exopolymer particles (TEP), aromatic amino acids, and humic DOM, with an elevated protein : humus ratio that suggested a higher contribution of labile components in viral-produced DOM than in algal exudates. At the natural community level, these results suggested that viral lysis could decrease the organic matter sedimentation and promotes respiration and nutrient retention whereas, in contrast, the enhanced TEP production could stimulate particle aggregation and export out of the water column. Based on metabolomics approach, Ankrah et al. (2014) demonstrated that phage infection of *Sulfitobacter* sp. redirects 75% of nutrients into virions, and 71% of 82 intracellular metabolites were significantly elevated in infected hosts, which also exhibited an elevated metabolic activity compared to non-infected populations. In contrast, more than 70% of 56 compounds in viral lysate decreased in concentration relative to uninfected controls, suggesting that small, labile nutrients from viral lysis are utilized quickly by non-infected cells. Ankrah et al. (2014) conclude that virus-infected cells are physiologically different from their uninfected counterparts, a situation which can alter the ecosystem biogeochemistry. One of the intrinsic mechanisms for that is the viral control of bacterial growth efficiency over a broad range of values (from 0.1 to 70%) and the related patterns in carbon and nutrient fluxes mediated by bacteria in pelagic environments (Bonilla-Findji et al., 2008; Motegi et al., 2009; Pradeep Ram et al., 2013).

There are many indirect ways that viruses can affect the biogeochemical cycling. Lytic viruses may shape the global climate by inducing the release of dimethyl sulfide (C_2H_6S), a gas known to influence cloud nucleation (**Figure 1**), which is massively produced by major bloom-forming species such as *M. pusilla*, *E. huxleyi*, and *Phaeocystis pouchetii* (Evans et al., 2007). Viral lysis of microorganisms in sinking aggregates could also decrease the sinking rate by alleviating the aggregates via release of trapped dissolved and colloidal materials. Alternatively, virus-infected cells could sink faster compared to non-infected cells (Lawrence and Suttle, 2004), contributing to the export of microbial cells downwards (**Figure 1**). In addition, viral lysis products contain polymers that can increase gel formation and affect the biological, physicochemical and optical properties of the sea water (Uitz et al., 2010), for example by generating aggregates and enhancing the departure of organic material from the euphotic zone (Lønborg et al., 2013). This can influence the amount of carbon exported to the deep ocean by the so-called biological pump, but also the Redfield stoichiometry of the water column, because the export of nutrients other than carbon needs to be balanced by new inputs (Mari et al., 2005). Hence, highly labile N- and P-nutrients contained in nucleic acids and amino acids, for example, will be used rapidly and retained in the euphotic layer, while the sinking particles will be abnormally rich in carbon, thereby increasing the efficiency of the oceanic biological pump activity (**Figure 1**; Suttle, 2007).

LYSOGENY: A SURVIVAL STRATEGY FOR VIRUSES

One of the key explanations for the omnipresence of viruses in natural ecosystems is undoubtedly through the existence of several lifestyles, of which two major pathways, namely lysis and lysogeny, are prevalent in aquatic systems (Pradeep Ram and Sime-Ngando, 2010). Lytic infections are by far the best studied of the virus–host interactions. Lysogenic activity has been less studied in aquatic environments where the temperate phage can alternatively integrate into the host genome as prophage. Prophages can be stable in their host for long periods of time, from months to years, with low probability of bacteriophages being released by spontaneous lysis (Paul, 2008). Examinations of natural prokaryotic communities inducible with a mutagenic agent (e.g., mitomycin C) have suggested that the fraction of lysogenic bacteria is typically <50% (range 0–100%) of the total abundance in marine environments. In freshwaters, these values fluctuate from 0 to 16% in temperate and tropical lakes, and from 0 to 73% in Antarctic lakes (Sime-Ngando and Colombet, 2009).

Lysogenic conversion has been described as a means of survival for viral populations that are threatened by poor host cell abundance and therefore cannot sustain population numbers through lytic infection alone (Stewart and Levin, 1984; Palesse et al., 2014). This situation occurs when the prokaryote abundance drops under a minimal threshold level, typically under about 10^5 cells ml^{-1} (Pradeep Ram et al., 2005). Microcosm experiments have recently demonstrated that nutrient addition in freshwater samples stimulates lytic viruses via enhanced growth rate of prokaryotes and, when limiting, rather promote lysogenic conversion (Pradeep Ram and Sime-Ngando, 2010). This finding was considered an explanation why lytic and lysogenic activities are often antagonistically correlated, supporting the idea that lysogeny may represent a maintenance strategy for viruses in harsh nutrient/host conditions which appeared as major instigators of the trade-off between the two viral lifestyles (Colombet et al., 2006). Both viral life cycles are thus apparently regulated by distinct factors, including environmental parameters (primarily resources for hosts) and host physiology for lytic cycle but

mainly host physiology for lysogenic cycle (Maurice et al., 2013; Palesse et al., 2014). It was also shown that the latter cycle is prevalent within the phylogenetic groups that dominate the whole bacterial community composition at a given time (Maurice et al., 2011).

VIRUSES AND MICROBIAL DIVERSITY

Viruses can impact microbial diversity and force diversification mechanisms toward host-cell evolution in two major ways (**Figure 1**). The first major way includes the direct effects of the intrinsic activities of viruses: (i) keep in check competitive dominants (i.e., lytic viruses), (ii) affect the metabolic properties of host cells which can acquire immunity to superinfections and new phenotypic and genotypic traits such as production of toxins (i.e., temperate phage conversion), and (iii) transfer both viral and host genes between species (transduction, GTA), thereby influencing speciation. The second major way comprises the indirect effects of viral activities such as (iv) the structuring effects of lysis products on species composition and richness, (v) the sustenance of the amount of information encoded in genomes that may favor horizontal gene transfer mechanisms, and (vi) the effects of physiological mechanisms involved in the resistance of host against viruses, through the host-pathogen arms race (**Figure 1**). Together with (i) the high abundance and broad geographical distribution of viruses and viral sequences within microbial fractions, and (ii) the prevalence of genes among typical viral sequences that encode microbial physiological functions, the above-mentioned effects establish environmental viruses as strong vectors that generate genetic variability of aquatic microorganisms and drive both ecological functions and evolutionary changes (Weinbauer and Rassoulzadegan, 2004).

VIRAL LYSIS PRODUCTS SHAPE EVOLUTIONARY TRANSITION, GLOBAL CARBON CYCLING AND THE DIVERSIFICATION OF MICROBIAL COMMUNITIES

Some viral groups such as the Caudovirales, the tailed double-stranded DNA phages, are probably older than the separation of life into the three now recognized domains of life (Ackermann, 1999; Hendrix, 1999). This suggests that, before the occurrence of eukaryotic grazers such as flagellates and ciliates, viruses were probably the main predators of cells in the prokaryotic world, and played a major role in the sophisticated forces (dispersal, competition, adaptive radiation, etc.) that shape biogeography and evolution. In contemporaneous marine systems, it was estimated that between 6% and 26% of the photosynthetically fixed carbon is channeled or "shunted" to the DOM pool by viral lysis of cells at all trophic levels (Wilhelm and Suttle, 1999). The carbon stored in the oceanic DOM pool equals that in atmospheric CO_2 (Hedges, 1992), suggesting that viral infection of marine prokaryotes and phytoplankton has an influence not only on global carbon cycling and climate but also on the microbial composition and community structure. A host density-dependent model, i.e., "the phage kills the winner" model (Thingstad and Lignell, 1997; Thingstad, 2000), was proposed based on the assumption that lytic viruses are highly specific to their host cells, at least at the species level. In this model, by eliminating the most competitive strains for resource acquisition, lytic viruses prevent dominance and increase niche availability for species-coexistence (**Figure 1**). This was recently tested by Motegi et al. (2013) who provide experimental data showing that viruses prevent the prevalence of taxa that were competitively superior in phosphate-replete conditions in NW Mediterranean surface waters. In addition, these authors obtained a statistically robust dome-shaped response of bacterial diversity to viral (VP) to bacterial (BP) production ratio, with significantly high bacterial diversity at intermediate VP:BP, corroborating the prediction from the general model that species diversity is maximized when productivity and disturbance are balanced.

LYSOGENS AND THE EFFECTS OF THEIR MUTUALISTIC VIRUSES

Virus-host interactions range in a gradient from true non-lethal parasitism (i.e., chronic infection) to fatal lytic infection, with intermediate mutualistic lifestyles (lysogeny, pseudolysogeny) where viral genomes stay within the host and confer new metabolic traits which can increase the fitness of the immune host but also the survival of the phage (**Figure 1**). A spectacular case for such a phage conversion is the finding that cholera infection is due to a lysogenic strain of the *Vibrio cholerae* bacterium. Since the toxin is encoded in the genome of the prophage and is not part of the host genome, non-lysogenic cells do not cause cholera (Weinbauer, 2004). In addition, it was recently shown that the type VI secretion systems in *V. cholerae* are virulence-associated proteins that are evolutionarily related to components of bacteriophage tails (Basler et al., 2012). Prophage induction events can change the bacterial community structure by increasing the diversity and richness of natural bacterial populations (Hewson and Fuhrman, 2007). For example, phage conversion can increase the fitness of cells (Edlin et al., 1975; Lin et al., 1977), which could influence community composition by allowing for the survival or dominance of such converted cells. Host immunization against infection by homologous phages and phage conversion are ways in which phages can influence microbial diversity in natural environments. More generally, it is likely that all living cells could contain active prophages in their genome. On average, 2.6 prophages have been detected per free-living bacterial species (Lawrence et al., 2002), and a number of bacterial genomes contain between 3 and 10% of DNA prophages (Brüssow and Hendrix, 2002). It was recently demonstrated that genes captured from ancestral retroviruses have been pivotal in the evolutionary acquisition of the key process through which most of the maternofoetal exchanges take place in placenta development in mammalian species (Dupressoir et al., 2009). This highlights the potential of phages as key players in the evolution and maintenance of living things.

LATERAL GENE TRANSFERS (LGTs) BY TRANSDUCTION: VIROMES ARE HUGE RESERVOIRS OF VIRALLY ENCODED HOST GENES THAT ARE CANDIDATES FOR LGTs

One of the most surprising findings of whole-genome sequencing is the enormous extent of lateral gene transfers. LGTs refer to the gene material exchanges between organisms that happen independently of reproduction (i.e., vertical gene transfers). General mechanisms include transformation (gene transfer by uptake of free genetic materials), conjugation (direct gene transfer from

cell-to-cell contact), the activity of GTAs, and transduction, where viruses are the main vectors that move nucleic materials from one cell to another (**Figure 1**). The transduction frequency in natural waters ranges from 10^{-8} to 10^{-5} per virus, and they might be up to 100 transductants l^{-1} day^{-1} (Jiang and Paul, 1998). An extrapolation exercise suggests that as many as 10^{24} genes are moved by transduction from viruses to hosts each year in the world's ocean (Rohwer and Thurber, 2009). This is considered an underestimate because of the action of the host DNA carrier GTAs (primarily in the α-Proteobacteria order Rhodobacterales) which are injected into recipient cells, providing a more efficient form of transduction (Lang et al., 2012). Although transduction is a random process, viruses can genetically alter microbial populations through lysogeny and transduction, and affect the flow of genetic information in aquatic ecosystems.

Large-scale metagenomics has shown that viruses contain diverse genes of interest, including virulence genes such as the cholera toxin genes, respiration, nucleic-acid, carbohydrate and protein metabolism genes, as well as genes involved in vitamin and co-factor synthesis, in stress response, and in motility and chemotaxis, which are more common in viromes (metagenomes of viruses) than in their corresponding microbiomes (metagenomes of microbes; Rohwer and Thurber, 2009). Microbes that take up these genes increase their competitive ability and extend their ecological niches (**Figure 1**). More interestingly, virally encoded host genes also include crucial photosynthetic genetic elements present in cyanophage genomes, which can be used to maintain the targeted function in dead hosts and accomplish the lytic cycle, and can be transferred between hosts as well (Lindell et al., 2005). About 10% of total global photosynthesis could be carried out as a result of phage genes originally from phages (Rohwer and Thurber, 2009). Given the prevalence of phage-encoded biological functions and the occurrence of recombination between phage and host genes, phage populations are thus expected to serve as gene reservoirs that contribute to niche partitioning of microbial species in aquatic ecosystems. Gene transfers by transduction may also represent an important mechanism for gene evolution in natural environments, and bacteriophage transduction could play an important role in contributing to the genetic diversity of microbial populations.

INDIRECT EFFECTS OF VIRUSES ON MICROBIAL DIVERSITY

The mass release of lytically infected cell contents can change the composition and the bioavailability of ambient organic substrates and nutrients, which are well known as key factors affecting the microbial composition and community structure (**Figure 1**). It has been shown that the presence of grazers in phosphorus-limited microcosms appeared to be a stimulating factor for prokaryotic growth and lytic viral proliferation, with a significant increase in the minor bacterial phylotypes as a consequence of the reduction of resource competition in prokaryotic assemblages (Sime-Ngando and Pradeep Ram, 2005). Prokaryotic phyla belonging to Bacteria, β-Proteobacteria and α-Proteobacteria responded significantly to lysis products, while Archaea and *Cytophaga-Flavobacterium* (now known as Bacteroidetes) rather changed their community size structure towards grazing-resistant forms (Pradeep Ram and Sime-Ngando, 2008, 2014). In general, the presence of grazers is a stimulating factor for prokaryotic growth and viral proliferation in the plankton, probably through nutrient regeneration process that increases niche availability and enhances prokaryotic diversity (Pradeep Ram and Sime-Ngando, 2008, 2014). The relative abundance, production and species richness of some bacterial phyla such as *Flectobacillus* or *Actinobacteria* increase more in the presence of both viruses and grazers that when only one of the consumers is present (Simek et al., 2007). Although, Weinbauer et al. (2007) reported both synergistic and antagonistic effects of viral lysis and protistan grazing activities on bacterial biomass, production, and diversity, and considered these effects as the result of group- or species-specific competition for prey and hosts, and the fact that both types of predators produce organic matter that potentially fuel growth.

Lysis products can contain phage-borne enzymes which can kill cells and also influence microbial composition (Fuhrman and Noble, 2000). Lysis products also include free genetic materials that can increase the amount of information encoded in genomes in the water column, and favor horizontal gene transfer mechanisms such as conjugation, transformation or genetic recombination (Weinbauer, 2004). Because all of the direct and indirect roles of viruses in LGTs ultimately result in "novel" genetic materials and information moving into the host cell, there are strong interactions between lateral and vertical gene transfer mechanisms. The acquisition of new genes can affect the genome size and the generation time of host communities and, perhaps more importantly, can move the strain or species barriers in microbial communities (Weinbauer and Rassoulzadegan, 2004). This can also affect the metabolisms and physiology of the host and, hence, their susceptibility to viral infection can be weakened up to a total resistance against viral infections. We are thus in the presence of an effective host-pathogen arms race for survival and coexistence, where host resistance is crucial for offspring and maintenance (**Figure 1**).

REISTANCE OF VIRAL HOST CELLS

Hosts and pathogens persist in the environment mainly through a molecular arms race between competing hosts and viruses where, as already discussed, viruses can affect their host in various ways, ranging from the enhancement of the host fitness and metabolic performances to mortality. Because parasites and pathogens tend to have shorter generation time than their hosts, they should evolve more rapidly and maintain advantage in the evolutionary race between defense and counter-defense. The paradox here is how do victim species survive and even thrive in the face of a continuous onslaught of more rapidly evolving enemies? One of the explanations is that the physiological, mechanical and behavioral costs of defense are lower compared to the cost of attack (Gilman et al., 2012). The viral host communities can thus respond to the pressure from their parasites and develop a sophisticated resistance shield, specific to each step in the viral cycle, but suffer from the cost of resistance which mainly includes a decrease in the fitness and growth rate or adaptive responses (review in Thomas et al., 2011), such as the production of proteins or extracellular matrices that mask the phage receptor (Bohannan and Lenski, 2000).

Viral receptors on the cell surface are complex families of proteins, carbohydrates or lipids, which serve normal physiological

functions of the cells but are hijacked by viruses for their adsorption. Host cell mutations and resistance to viral adsorption grossly include modifications of the receptor structure, alterations of receptor accessibility, decreases in the number of receptors, or loss of receptor sites. A spectacular example of host surface modification is the so-called "Cheshire Cat" strategy where the resistant haploid phase of the algal haptophyte *E. huxleyi* does not calcify and is "invisible" to viruses, in contrast to the susceptible diploid phase (Frada et al., 2008). Similarly, it was shown that colonial forms of the algal prymnesiophyte *P. pouchetii* are resistant to viruses because they are surrounded by an "outer skin," in contrast to individual cells (Brussaard et al., 2007; Jacobsen et al., 2007). Brussaard et al. (2005) demonstrated that the morphology (solitary versus colonial) of the prymnesiophyte *Phaeocystis globosa* differently regulate viral control of *P. globosa* bloom formation, depending on irradiance, nutrient, and grazing regimes. A modeling exercise suggested that the enhanced growth rates, the low viral infection rate, and the low grazing rate on cells in colonies, as compared to free-living single cells of *P. globosa*, result in a massive blooming of *P. globosa* colonies. When the controlling nutrient becomes depleted, the colonies disintegrate and liberate single colonial cells that are subject to high rates of viral infection and grazing (Ruardji et al., 2005). It is thus likely that cell hosts embedded in colonies are more resistant to viral infection than free-living cell hosts.

The host mechanisms of blocking viral replication and entry remain relatively unknown in marine organisms. Stolt and Zillig (1994) reported a prophage-encoded gene (*rep*) in the marine archaea *Halobacterium salinarum* able to protect cells from viral infection. Tomaru et al. (2009) have shown that viral genome replication in resistant cells of the dinoflagellate *Heterocapsa circularisquama* is repressed. Bacteria, cyanobacteria, and archaea are known to prevent viral replication by the acquisition of immune systems consisting of short fragments of foreign nucleic acids into clustered interspaced short palindromic repeats (CRISPRs) in their genomes. CRISPR spacers have homologies with mobile genetic elements such as bacteriophages and plasmids, and those identical to phage sequences provide resistance against viral infection (Thomas et al., 2011). Overall, the various effects of viruses force costly resistance mechanisms in their host communities, where the co-existence of sensitive and resistant host cells is likely a result of a trade-off between competitive ability and mortality. Lennon et al. (2007) provided a nice example of the ability of marine cyanobacteria *Synechococcus* to evolve resistance with fitness costs associated with the identity of a few particular viruses, suggesting that variability in fitness costs associated with viral resistance can structure microbial communities and regulate biogeochemical cycles.

VIRUSES AS CELL PARTNERS AND CELL MANIPULATORS

Although viruses are most often studied as pathogens, many are beneficial to their hosts, providing essential functions in some cases and beneficial functions in others. For example, many pathogenic bacteria produce a wide range of virulence factors that help them to infect their hosts. There are numerous examples of such virulence factors that are expressed not from the bacterial genome but from a phage genome, such as diphtheria, Shiga, and cholera toxins (Brüssow et al., 2004). The nuclei of dinoflagellates contain permanently condensed, liquid crystalline chromosomes that seemingly lack histone proteins, and contain remarkably large genomes. The molecular basis for this organization was recently provided by Gornik et al. (2012) who discovered that histone proteins were replaced, during evolutionary events, by a novel, dominant family of nuclear proteins (called DVNPs, dinoflagellate/viral nucleoproteins) that is only found in dinoflagellates and, surprisingly, in a family of large algal viruses, the Phycodnaviridae. The authors concluded that gain of a major novel family of nucleoproteins from an algal virus occurred early in dinoflagellate evolution and coincided with rapid and dramatic reorganization of the dinoflagellate nucleus.

Although few examples for viral manipulators were reported in the microbial world, studies from eukaryotes have shown that some viruses are essential for the survival of their hosts; others give their hosts a fighting edge in the competitive world of nature, while some others have been associated with their hosts for so long that the line between host and virus has become blurred (Roossinck, 2011). Some virologists think that modern genomes are essentially remnants of ancient viruses. Intact and fragmented retroviruses are found in the genomes of almost all eukaryotes. Approximately 8% of the human genome is derived from retroviruses (Lander et al., 2001), and this percentage increases dramatically if other mobile genetic elements are included (Kazazian, 2004). At least some endogenous retroviruses encode functional genes and are thought to be involved in major evolutionary leaps. For example, the evolution of placental mammals probably occurred after the endogenization of a retrovirus. Retroviral envelope proteins cause fusion of cell membranes, a process that not only allows the invasion of oncogenic viruses but also is required for the development of the placental syncytium, an essential part of the barrier that prevents maternal antigens and antibodies getting into the fetal bloodstream and provoking abortion (Dunlap et al., 2006). All living things thus have something of viruses in their genomes, some of which may be beneficial to their fitness and evolution.

Furthermore, recent researches are increasingly demonstrating the ecological and evolutionary importance of viruses of symbiotic organisms within their hosts, in complex tripartite interactions. A famous example is the existence of an endogenous virus that (i) alter the life story of the sea photosynthetic slugs *Elysia chlorotica* by possibly synchronizing the lifetime of the stolen chloroplasts to that of the slugs, (ii) and is probably the vector of the horizontal gene transfer between the slug and the chloroplasts that provides 80–90% of the genes necessary for photosynthesis (Rohwer and Thurber, 2009). However, recent analysis of the genome of *E. chlorotica* egg DNA provides no clear evidence of horizontal gene transfer into the germ line of this kleptoplastic mollus, and suggests that algal nuclear genes or gene fragments are present in the adult slug (Bhattacharya et al., 2013). Polydnaviruses (PDVs) are viruses associated with wasp species that parasitize lepidopteran larvae. PDV particles are injected along with the eggs of the wasp into the lepidopteran larvae (or eggs) and express proteins that interfere with host immune defenses, development, and physiology; this interference enables wasp larvae to survive and develop within the host. It was recently shown that the PDV particles are well conserved in the braconid wasp ovaries and originated from the integration of nudivirus

machinery into the genome of an ancestral wasp about 100 million years ago. Bézier et al. (2009) found that nudiviral genes themselves are no longer packaged but are actively transcribed and produce particles used to deliver genes essential for successful parasitism in lepidopteran hosts. Recent works in the field of entomology have revealed tripartite associations between insects, bacteria, and phages, where phages control bacteria which affect the physiology and ecology of the host. Overall, it is clear that viruses are key players in complex symbiotic associations between animals and their sequestered plant chloroplasts, parasitoid insects and their insect hosts, and between bacterial pathogens and their insect hosts. This is an overlooked role of environmental viruses that probably occurs in all habitats where viruses vector important traits, such as defense against or sensibility to parasitoids, within and among symbionts of animal and probably plant host lineages.

CONCLUDING REMARKS

Aquatic viral ecology is a relatively recent discipline, in increasing development. Viruses are omnipresent in aquatic environments, including the most extreme and worst biotopes, where they often represent the most abundant biological entity. Because all types of cell in the three domains of life have their specific viruses and offer ecological niches to different viral lifestyles, viruses are considered the greatest reservoir of the uncharacterized biological diversity on the earth, which is being probed and described at an increasingly rapid rate, almost exclusively with molecular sequence data. For example, Hewson et al. (2013) recently used a metagenomic approach to identify circular, single-stranded DNA viruses that may be involved in the seasonal dynamics of *Daphnia* spp. in Oneida and Cayuga lakes (upstate New York). Because *Daphnia* plays a critical role in many lake ecosystems, such viruses may have important effects on herbivory and thus carbon flow through the lake ecosystem.

Our conceptual understanding of the function and regulation of aquatic ecosystems, from microbial to global biogeochemical processes, has changed with the study of viruses. Viral-mediated prokaryotic mortality roughly equals bacterivory from protists, which is a significant departure from the traditional view that predation and resource availability are the main factors controlling prokaryotic abundance and production in pelagic systems. Viruses influence both the retention and the export of organic matter in the pelagic realms. Given the prevalence of phage-encoded biological functions within host cells and the occurrence of recombination between phage and host genes, phage populations serve as gene reservoirs that contribute to niche partitioning of microbial species in aquatic ecosystems. Viral-mediated gene transfers include diverse mechanisms (transduction, transformation, conjugation, and recombination) that are known to affect gene evolution in the marine environment. It is thus clear that most of the viruses are not pathogens but mutualistic cell partners that provide helper functions. The discovery of giant viruses of eukaryotes (absent in this review) which encode trademark cellular functions has weakened the gap between inert and living things. Overall, studies in aquatic viral ecology are sources of novel knowledge related to the biodiversity of living things, the functioning of ecosystems, and the evolution of the cellular world. The future challenge is to extend viral ecology studies (i) to all biotopes in the biosphere such as lakes, rivers, underground waters, soils, clouds, air, etc., (ii) to all living organisms which are susceptible to viral attacks (e.g., zooplankton, Archaea etc.), and (iii) the related functions, some of which are crucial to global change (e.g., consumption and production of greenhouse gas such as methane etc.), and (iv) to multi-partner symbioses and their effects on the food web dynamics and host maintenance and adaptive evolution.

More generally, next-generation sequencing technologies are increasingly revealing that microbial taxa likely to be parasites or symbionts are probably much more prevalent and diverse than previously thought. Every well-studied free-living species has parasites; parasites themselves can be parasitized. As a rule of thumb, there is an estimated four parasitic species for any given host, and the better a host is studied the more parasites are known to infect it. Therefore, parasites and other symbionts should represent a very large number of species and may far outnumber those with "free-living" lifestyles. Paradoxically, free-living hosts, which form the bulk of our knowledge of biology, may be a minority. Microbial parasites offer good experimental models because they are typically 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. Extensive studies on all aspects of parasites and other symbionts in aquatic microbial ecology are warranted, including method development, life cycle, interactions with hosts and competing microbes, coevolution, effects on food webs, and biogeochemical cycles. I believe that including viruses in the more complex world of parasites and symbionts is promising for biology and ecology in the future.

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Abalone farm discharges the withering syndrome pathogen into the wild

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An intracellular bacterium Candidatus *Xenohaliotis californiensis*, also called Withering-Syndrome Rickettsia-Like Organism (WS-RLO), is the cause of mass mortalities that are the chief reason for endangerment of black abalone (*Haliotis cracherodii*). Using a real-time PCR assay, we found that a shore-based abalone farm (AF) in Santa Barbara, CA, USA discharged WS-RLO DNA into the ocean. Several other shore-based AFs discharge effluent into critical habitat for black abalone in California and this might affect the recovery of wild black abalone. Existing regulatory frameworks exist that could help protect wild species from pathogens released from shore-based aquaculture.

Keywords: abalone, aquaculture, emerging disease, endangered species, fisheries, pathogen, water quality

INTRODUCTION

Black abalone (*Haliotis cracherodii*) were once stacked three deep in the California intertidal (Cox, 1960). But in the mid-1980s, marine biologists noticed black abalone disappearing from their study plots (Haaker et al., 1992). Lying next to the empty quadrats were dying abalone, their muscular foot withered and unable to remain attached to the rocks. These "withering syndrome" die offs were often rapid and extensive (Richards and Davis, 1993). At first, the die offs were attributed to an El Niño Southern Oscillation event (Davis et al., 1992), but the continued spread of mass mortalities was more indicative of an infectious process (Lafferty and Kuris, 1993). Pathologists discovered the cause: a novel intracellular bacterium Candidatus *Xenohaliotis californiensis*, or Withering Syndrome Rickettsia-Like Organism (WS-RLO; Friedman et al., 2000). Withering syndrome spread north and south from the Santa Barbara Channel Islands over the next two decades throughout most of the black abalone's range (Lafferty and Kuris, 1993; Altstatt et al., 1996). WS-RLO also infects and impacts, to various degrees, the six other California abalone species (Friedman et al., 2002). The pathogen can spill over from more resistant abalone species, which could contribute to the decline of less resistant species such as black abalone (Ben-Horin, 2013). Locally extirpated from most of its range with no sign of population recovery, the National Marine Fisheries Service listed the black abalone as endangered in 2009 (Neuman et al., 2010). With no commercial fishery, prices for red abalone (*Haliotis rufescens*) increased and shore-based abalone farms (AF) grew and sold four to 5-year-old juvenile red abalone. By 2008, production was dominated by 3–4 farms (Moore and Moore, 2008) and had risen to ∼227 tonnes (US $8–9 million), a production level that remains true today.

Even though the young abalone were soon infected with WS-RLO in culture, the prolonged incubation period and temperature dependence of clinical withering syndrome (Moore et al., 2011) allows farms to keep infected abalone alive to market size with minimal losses. Farms in cooler regions, such as central California, suffer lower losses despite the presence of the pathogen (Friedman and Finley, 2003). Pathogen export from AFs has two precedents that motivated our study. In the early 1990s, California AFs became infested with a sabellid polychaete worm (*Terebrasabella heterouncinata*, Fitzhugh and Rouse, 1999) that abalone farmers introduced along with abalone from South Africa. The worms live in snail shells and their presence slows growth (Kuris and Culver, 1999). Shell debris exiting the outflow of The Abalone Farm at Cayucos, California, led to the infestation of native turban snails (Kuris and Culver, 1999). However, counter to most species introductions, the sabellid-worm invasion was halted (Culver and Kuris, 2000). When the discharge was discovered, volunteers removed 1.6 million snails from around the farm, ceasing transmission in the wild. The AF then cleaned up its stock, screened its outflow, and stopped dumping shell debris into the intertidal. The second precedent is from Australia where, in 2005, a herpes virus that causes Abalone Viral Ganglioneuritis led to mortalities in AFs in Victoria. In 2006, the disease emerged in wild abalone near farms where it has spread along the coast, leading to substantial fisheries losses (Hooper et al., 2007); nowhere is the conflict between aquaculture and wild fisheries more clear (and litigious). Global declines in wild fisheries and the rise in demand for seafood for human consumption have driven the rapid growth of aquaculture, particularly for expensive foods like abalone (Naylor et al., 2000). Growth in aquaculture may reprieve pressure on wild fisheries; however, ecological risks such as the spread of

infectious diseases from farms to wild stocks have uncertain and often negative impacts on wild populations (Daszak et al., 2001). Understanding the indirect impacts of aquaculture is therefore critical for the sustainable management of disease-impacted fisheries. Here we asked whether the WS-RLO pathogen was being exported from an abalone aquaculture facility near Santa Barbara, CA, USA. A real-time, quantitative polymerase chain reaction (qPCR) test for the pathogen in water samples indicated high WS-RLO DNA densities at the farm's outflow that dissipated with distance.

MATERIALS AND METHODS

Seawater samples were collected for assessment of WS-RLO in the western Santa Barbara Channel (CA, USA) during August 2011. We first collected three 100 ml samples directly from the effluent of the AF. We then collected 36 100 ml seawater samples immediately offshore the AFs at Dos Pueblos (DP) and again at Hope Ranch-More Mesa (HR-MM), located 20 km ESE of the farm. Both near shore sites contain patchy rocky subtidal habitat within soft bottom substrate. At both sites, 18 of the 36 samples were collected at the surface and the remaining samples were collected at a depth of approximately 5 m. We collected 9 of the 18 surface and subtidal samples at both sites over rocky habitat and the remaining nine samples were collected over soft bottom habitat. After collection, the seawater samples were placed on ice in a cooler and kept in the dark until further processing in the laboratory. Seawater samples were filtered onto 47 mm diameter, 0.2 μm-pore size, Supore® filters (Pall Corp., Port Washington, NY, USA). We rinsed the filter apparatus with 10% bleach between each sample to prevent carry-over of WS-RLO, and filtered four, 100 ml artificial seawater samples as negative controls (negative filter controls). Filters were folded and placed immediately into microcentrifuge tubes containing 200 μl of Qiagen lysis buffer with proteinase K (Qiagen, Santa Clarita, CA, USA). We isolated DNA from the filters using a Qiagen DNeasy Stool Kit following the manufacturer's protocol, which included a final elution of the DNA from the column using 100 μl of the elution buffer AE (Qiagen). We quantified the density of WS-RLO DNA in seawater samples by real-time SYBR Green® PCR on the Applied Biosystems 7500 Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA), using the primer pair RA5-1 and RA3-6 specific to a 160 bp fragment of the WS-RLO 16S rDNA (GenBank Accession number: AF133090) described by Andree et al. (2000). The WS-RLO specific primer mix was prepared manually by diluting each 100 μM primer (Life Technologies) to 9 μM. Amplifications were done in 20 μL reaction mixtures with 20–50 ng of genomic DNA measured spectrophotometrically. Reagents were added in the following proportions: 10 μL Fast SYBR Green Master Mix with AmpliTaq® Fast DNA Polymerase (Life Technologies), 2 μL RA5-1, 2 μL RA3-6, 4 μL nuclease-free H_20 (Life Technologies), and 2 μL of template DNA for a 20 μL reaction volume. The thermal-cycling conditions were as follows: initial polymerase activation for 20 s at 95°C, followed by 50 cycles of 3 s at 95°C, and 30 s at 60°C.

Quantification of the amplified product was measured on a cycle-by-cycle basis via the acquisition of a fluorescent signal generated by the binding of the SYBR Green® fluorophore to double stranded DNA. Our interest was in the relative differences in concentrations of WS-RLO DNA among sampling locations, and therefore used a relative standard curve for DNA quantification as follows. First, WS-RLO DNA was isolated from post-esophagus tissue of three WS-RLO positive red abalone using a Qiagen DNeasy tissue kit following the manufacturer's protocol. The obtained DNAs were pooled to provide a 1× relative density sample, then 10-fold serially diluted using the elution buffer AE (Qiagen). We prepared three assays of 20 replicates of each relative density standard (D_S) and these relative standard samples were analyzed by real-time PCR. The 7500 Fast System software fits a standard curve by regression of D_S on the threshold cycle number, C_t, at which the fluorescence signal (ΔR_n) crosses a value exceeding the background fluorescence. This C_t value is proportional to the logarithm of the target DNA concentration in the assay. We set the threshold value of ΔR_n to 0.1, based on previous runs of the 7500 Fast System. We determined mean values of C_t, as well as the associated standard deviations, for all values of D_S. The sensitivity of real-time PCR assays to differences in initial template amounts increases at low concentrations of template DNA, and we therefore defined the limit of detection (LoD) as the minimum value of D_S where ≥50% of the test runs amplified and the standard deviation of estimated C_t values ≤ 0.5 (OIE-World Organisation for Animal Health, 2009). Unknown seawater samples were run in triplicate, and all runs included triplicate relative standards, two negative filter controls and two negative plate controls (nuclease-free H_2O in lieu of DNA template). The 7500 Fast System software automatically plots the relationship between relative density and C_t and converts C_t values of unknown samples to relative densities using the obtained regression formula. We confirmed the specificity of all amplified products by melting curve analysis. Precision, or reproducibility of the real-time SYBR Green® assay, was estimated in a separate experiment. Reproducibility can vary within an assay (intra-assay variability), due to reaction-to-reaction variance in pipetting volumes and measurement among wells, as well as between assays (inter-assay variability) due to variance arising from slight differences in reaction components. We tested the precision of the real-time assay in three separate runs, sampling five replicates of one WS-RLO positive seawater sample and the 1× relative standard sample in each run. We sampled intra-assay variability as the standard deviation of the estimated C_t values within each run, and tested the equality of variances between runs using Levene's tests. We tested for inter-assay variability using ANOVA. We estimated the relative concentration index (CI_R) for all seawater samples as the mean of obtained values of relative density among triplicate samples, and tested for differences in CI_R among the habitat and depth covariates, as well as among the sampling sites by Kruskal–Wallis analysis of variance by ranks. All statistical analyses were performed in Matlab® version 8.1.0 (The Mathworks, Inc., Natick, MA, USA).

RESULTS

The assay was able to detect dilute WS-RLO DNA in relative standard and seawater samples, and both filter and plate negative controls tested negative for WS-RLO DNA. We observed amplification in ≥50% of the standard samples across seven orders

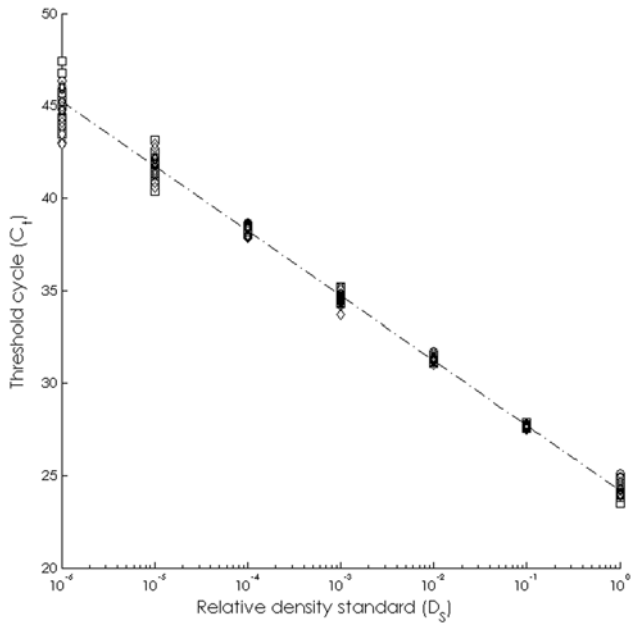

FIGURE 1 | Standard curve representing the relationship between the relative concentration of the WS-RLO DNA (D_S) and the threshold cycle (C_t) determined by the real-time qPCR assay.

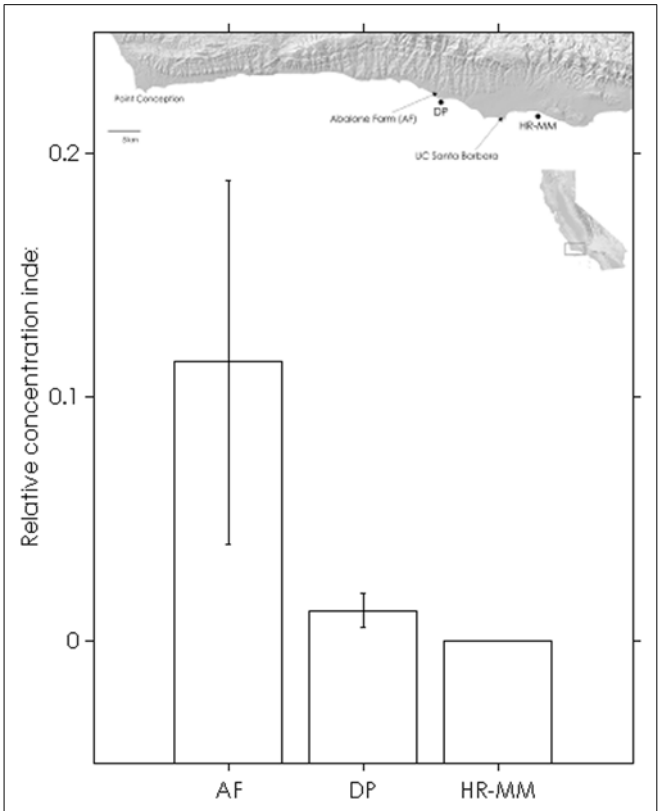

FIGURE 2 | Means (±SE) of WS-RLO DNA relative concentration indices at the abalone farm (AF), Dos Pueblos (DP), and Hope Ranch-More Mesa (HP-MM), with a map of the sites. Long-shore current flows from left to right. The index describes the concentration of WS-RLO DNA in seawater relative to pooled sample of WS-RLO DNA taken from the post-esophagus of WS-RLO infected red abalone with >100 WS-RLO inclusions per 200× magnification field of view.

of magnitude in the three replicate assays (**Figure 1**), and all but three points fell within the 95% confidence interval of C_t estimated for each relative standard. The standard deviation of C_t values in the 0.00001× and 0.000001× standards exceeded the *a priori* threshold value of 0.5 however, and we therefore used a detection limit of 0.0001× (mean C_t = 38.27 cycles). The efficiency of the assay, estimated from the slopes of the three replicate standard curve regression equations ranged from 92 to 93%. The assay was repeatable enough to give us confidence that variation among samples was attributed to variation in the signal. For all intra-assay variability tests of real-time PCR assay precision, the standard deviation of the estimated C_t values within each run was ≤0.15. We did not find significant differences in the variability of estimated C_t values between runs, for both the 1× relative standard sample (Levene's test; $P = 0.34$) and positive seawater sample (Levene's test; $P = 0.38$). Estimated C_t values did not differ between runs, both for the 1× relative standard sample (one-way ANOVA; $F_{2,14} = 1.41$, $P = 0.28$) and the positive seawater sample (one-way ANOVA; $F_{2,14} = 0.66$, $P = 0.54$). WS-RLO DNA dissipated with distance from the farm. All seawater samples taken at the farm effluent contained WS-RLO DNA. Seventeen of 36 samples taken immediately offshore the farm at DP contained WS-RLO DNA, and only one of 36 samples taken at Hope Ranch/More Mesa contained WS-RLO DNA. We did not observe significant associations between relative concentration indices and habitat (Kruskal–Wallis test; $H = 0.54$, df = 1, $P = 0.63$) and depth (Kruskal–Wallis test; H = 3.48, df = 1, $P = 2.21$). However, relative concentration indices differed significantly among the sampling locations (Kruskal–Wallis test; H = 77.18, df = 2, $P < 0.001$), and were greatest at the farm outflow and dissipated in the seawater offshore from the aquaculture facility (**Figure 2**). WS-RLO DNA was detectable, but very dilute at Hope Ranch/More Mesa.

DISCUSSION

Our results show that outflow from a California abalone aquaculture facility contains WS-RLO DNA. This DNA also occurs offshore of the outflow and is distributed throughout the water column over sand and rock habitat. The DNA is dilute, but detectable for almost 20 km to the east (with the long-shore current). There are other potential sources of the dilute WS-RLO DNA detected in our eastern sampling site. Wild abalone could exist there undetected and several dozen infected abalone are housed in the flow-through sea water system at the University of California, Santa Barbara (UCSB), CA, USA, though outflow from these abalone is not discharged directly into the ocean (see below). How WS-RLO discharge affects black abalone recovery depends on whether AFs are near good black abalone habitat. Black abalone are most common at exposed rocky shores with vertical rock faces and crevices (Cox, 1960). This habitat is abundant in northern Santa Barbara County, and on the offshore Channel Islands. However, due to low relief rocky habitat and frequent burial by sand, long-time UCSB collector Shane

Anderson (personal communication) only saw black abalone regularly at two places in our study area: in the mid 1970s to mid 1990s there were three, >10 cm black abalone on the most offshore rock at Campus Point (near the campus seawater intake), and black abalone were common nestled amongst the mussels on the Ellwood Pier (4 km east of the Cultured AF) until the mid 1990s. Overall, these observations suggest that the area near the AF we sampled is not particularly good black abalone habitat. In fact, southern Santa Barbara County was not designated as black abalone critical habitat. Although the AF we sampled is not located in critical habitat for black abalone, there are AFs in black abalone critical habitat. If these farms also discharge WS-RLO, they might prevent black abalone from recolonizing into critical habitat. The only way to understand the magnitude of pathogen discharge is to test the outflow of other facilities that house abalone. A complication in determining the impact of pathogen discharge is the recent discovery of a phage that infects the WS-RLO (Friedman and Crosson, 2012). This phage appears to be common in AFs and could explain reductions in the impact of WS-RLO on farmed abalone. The export of phage from AFs might also help reduce the impact of WS-RLO on wild abalone, though this remains a hypothesis. The impact of the WS-RLO from AFs also depends on how far currents can transport live, viable WS-RLO from the discharge. The WS-RLO can likely survive in seawater for several days based on the spread of the bacterium and osmolarity of host cells that is similar to that of seawater (C. Friedman pers. comm.). Ocean currents in the western Santa Barbara Channel have a general counterclockwise circulation, and therefore effluent from the sampled AF, as well as any presumptive pathogens contained within the effluent, flows toward critical habitat at San Miguel Island with an expected 80 km transit in about 5 days (Beckenback, 2004). Using the qPCR assay described, it should be possible to predict the advection and dilution of WS-RLO with distance from this and other AFs, and therefore quantify the indirect impact of aquaculture on wild abalone stocks. However, given that wild infected abalone may be present and that the qPCR assay tests for presence of target DNA and not the presence of a viable organism (Burreson, 2008), these data should be used to inform transmission trials needed to confirm this hypothesis. The qPCR assay, however, serves as a proxy to assess potential risk. Regulators and environmental advocates have not considered the potential impact of WS-RLO discharge from AFs, research institutions, or public aquaria. For instance, the Monterey Bay Aquarium's Seafood Watch program labeled California farmed abalone as sustainable seafood because the main known environmental effects were assumed to be minor now that farms no longer export sabellid worms. For AFs outside of black abalone critical habitat, farm discharge may not present an impact requiring management (depending on how far the WS-RLO can travel). For AFs that discharge into critical habitat, one possible management option is to eliminate the pathogen from the system by using antibiotics to treat stock and prevent re-infection (Friedman et al., 2003). Although this method is labor intensive, treatment with the antibiotic oxytetracycline has shown to provide long-term protection from WS-RLO infection (Friedman et al., 2007), and therefore provides a feasible management option for small to medium-sized farms, public aquaria, and research institutions. An alternative to antibiotic treatment would be to divert the discharge from the ocean. For instance, outflow could be sent to a sewer or to holding ponds (e.g., artificial wetlands) instead of to the ocean. As an example, relative concentrations of WS-RLO DNA at our abalone facility at UCSB are comparable to the relative concentrations of WS-RLO DNA we observed at Hope Ranch – More Mesa (observed CI_R have ranged from 0 to 0.0002). To diminish the chance that WS-RLO will enter the open ocean, the outflow from our abalone facility is directed into the Campus Lagoon, an artificial estuary-like pond closed to tidal flushing. Another approach would be to treat the discharge with heating, chemicals, filtration, or UV sterilization. Further research could help assess the cost and benefits of each approach. An obvious question is if and how to regulate the discharge of WS-RLO. Whether or not to regulate is a policy decision that we will not advocate for here. In California, there are many agencies involved in fisheries and coastal development. The California Department of Fish and Wildlife (CDFW) can prohibit aquaculture discharge or place restrictions or requirements on aquaculture operations where it is determined aquaculture activities are detrimental to adjacent native wildlife. The California National Pollutant Discharge Elimination System (NPDES) considers that dischargers of point source pollutants shall not cause degradation of indigenous biota, and marine communities. The National Marine Fisheries Service (NMFS) could choose to determine that aquaculture discharge of WS-RLO jeopardizes the continued existence of endangered black abalone. Finally, construction of AFs requires a coastal development permit by the California Coastal Commission and this state agency has the flexibility to consider impacts to marine biological resources when granting permits. Decisions to enact this regulatory framework require information about potential impacts, such as we have described above. Our results focus on black abalone, but these general issues apply to aquaculture projects around the world, including for finfish. For example, wild juvenile salmon can be impacted by sea lice as they swim by salmon farms (Krkosek et al., 2006). Similarly salmon farms may have increased bacterial kidney disease (Jude and Leach, 1999). Regulators might specifically consider whether discharge from aquaculture facilities includes pathogens that can affect wild species, particularly those that are in danger of extinction.

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Reduced disease in black abalone following mass mortality: phage therapy and natural selection

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Black abalone, *Haliotis cracherodii*, populations along the NE Pacific ocean have declined due to the rickettsial disease withering syndrome (WS). Natural recovery on San Nicolas Island (SNI) of Southern California suggested the development of resistance in island populations. Experimental challenges in one treatment demonstrated that progeny of disease-selected black abalone from SNI survived better than did those from naïve black abalone from Carmel Point in mainland coastal central California. Unexpectedly, the presence of a newly observed bacteriophage infecting the WS rickettsia (WS-RLO) had strong effects on the survival of infected abalone. Specifically, presence of phage-infected RLO (RLOv) reduced the host response to infection, RLO infection loads, and associated mortality. These data suggest that the black abalone: WS-RLO relationship is evolving through dual host mechanisms of resistance to RLO infection in the digestive gland via tolerance to infection in the primary target tissue (the post-esophagus) coupled with reduced pathogenicity of the WS-RLO by phage infection, which effectively reduces the infection load in the primary target tissue by half. Sea surface temperature patterns off southern California, associated with a recent hiatus in global-scale ocean warming, do not appear to be a sufficient explanation for survival patterns in SNI black abalone. These data highlight the potential for natural recovery of abalone populations over time and that further understanding of mechanisms governing host–parasite relationships will better enable us to manage declining populations.

Keywords: abalone, *Haliotis*, withering syndrome, rickettsial, endangered, histology, selection, phage

INTRODUCTION

The black abalone, *Haliotis cracherodii*, was once abundant along rocky shores of the NE Pacific (Haaker et al., 1986; Geiger, 1999). In some locations, such as the California islands, black abalone were often stacked 4–5 deep (Douros, 1987). Black abalone populations supported indigenous subsistence fisheries for at least nine millennia (e.g., Erlandson et al., 1996) and, more recently, supported a relatively stable commercial harvest (Karpov et al., 2000) until 1982 when a strong El Niño Southern Oscillation (ENSO) preceded catastrophic population declines (Dayton and Tegner, 1984). Near extirpation of black abalone was documented at many sites (up to 99% losses; Tissot, 1991, 1995; Haaker et al., 1992; VanBlaricom et al., 1993; Altstatt et al., 1996; Friedman et al., 2000; VanBlaricom et al., 2008; Neuman et al., 2010; Blaud, 2013). Due to precipitous declines, the black abalone became the second abalone species to be listed as "endangered" pursuant to the US Endangered Species Act (ESA) of 1973 as amended (16 US Code §§1531-1543 et seq.) on January 14, 2009 (74 US Federal Register 1937). Concern over the ability of depleted populations of black abalone to recover following mass mortality was heightened by the fact that recruits have been shown to largely originate from local stocks (Hamm and Burton, 2000; Chambers et al., 2005, 2006).

The post-ENSO mass mortalities resulted from an infectious disease called withering syndrome (WS). The etiological agent of WS is an intracytoplasmic rickettsia-like organism (WS-RLO), "*Candidatus* Xenohaliotis californiensis," which infects abalone gastrointestinal epithelia (Friedman et al., 2000). WS has also impacted populations of green, *H. fulgens*, and pink abalone, *H. corrugata*, in Mexico (del Álvarez et al., 2002) and contributes to seasonal losses of cultured red abalone, *H. rufescens*, in California, particularly during ENSO conditions (Moore et al., 2000; Braid et al., 2005; Vilchis et al., 2005). The endangered white abalone (*H. sorenseni*) is known to be vulnerable to mortality caused by WS in captivity, but population-scale effects in natural habitats are unknown (Friedman et al., 2007; Crosson et al., 2014). WS is now endemic in California and is an impediment to recovery of affected abalone species (Moore et al., 2002; Friedman and Finley, 2003; Friedman et al., 2007).

Infections with the WS-RLO begin in the posterior esophagus (PE) epithelium and cause morphological changes (metaplasia) in the digestive gland (DG) of the abalone host that enable the bacterium to infect this organ (a secondary target tissue for the WS-RLO; Friedman et al., 2000) and disrupt its function leading to catabolism of the foot muscle to obtain energy and, finally, death (Friedman et al., 2000; Braid et al., 2005). Clinical disease occurs in RLO-infected animals during periods of elevated seawater temperatures (e.g., ≥18°C; Moore et al., 2000; Friedman et al., 2002; Braid et al., 2005; Vilchis et al., 2005). However, a

recent study comparing the influence of ENSO with La Niña and ambient ("normal") temperature conditions illustrated that the thermal threshold for WS may be lower than 18°C (Moore et al., 2011). Thus the severity and spread of WS is linked to climatic variation (temperature). In black abalone, temperature variation was shown to increase susceptibility to infection, while high mean temperatures increase mortality rates of infected individuals (Ben-Horin et al., 2013).

Some black abalone survived the post-ENSO mass mortalities, leading to the speculation that remaining individuals might be somehow less affected by infection (Lafferty and Kuris, 1993). With this possibility in mind, the black abalone fishery was closed in 1993 to protect whatever genetic variation remained [California Department of Fish and Game (CDFG), 1993]. From 95 to 99% of black abalone in nine permanent intertidal study sites at San Nicolas Island (SNI, which is located off Southern California; VanBlaricom et al., 1993) died within 10 years of WS's emergence at the island in spring 1992 (VanBlaricom et al., 1993). All study sites experienced mass mortalities, but beginning in 1996 it was apparent that survival rate was higher at study site 8 than at other SNI study sites (Chambers et al., 2005, 2006; Crosson et al., 2014). Subsequently, recruitment of black abalone was observed on SNI beginning in 2002 and was especially marked at site 8 (Chambers et al., 2005, 2006). A shift in climate trend to a "hiatus" in warming over the past decade (e.g., Easterling and Wehner, 2009; Kosaka and Xie, 2013) may have contributed to observed recruitment but did not appear to fully explain this change. In particular, an ENSO event that occurred in 2004–2005 in Southern California (Ben-Horin et al., 2013) and the fact that recruitment on SNI has occurred primarily in one locale suggest that temperature alone is not driving recovery of black abalone on SNI (Crosson et al., 2014). These observations led to the hypothesis that the young abalone on SNI might be progeny of parents with heritable traits that had helped them survive the past mass mortality events.

To test this hypothesis, we undertook studies to characterize differential susceptibility or resistance to WS of newly emergent recruits among populations of black abalone using controlled laboratory challenges. In the absence of acquired immunity, there are three ways that hosts might evolve in response to a pathogen: decreased susceptibility (inability to become infected), increased resistance (control of the pathogen once infected), or increased tolerance (lack of disease despite infection; Boots and Bowers, 1999). We formulated specific predictions for these three hypotheses. If past exposure had selected for reduced susceptibility, we predicted that abalone from SNI (disease-selected abalone) would be less likely to become infected when exposed to the WS-RLO compared with abalone from Carmel (naïve abalone). If past exposure had selected for resistance, we predicted that selected abalone would have lower-intensity infections in one or both target organs (PE and DG) after a successful exposure compared with naïve abalone. If past exposure had selected for tolerance, we expected selected abalone would show a weaker relationship between RLO intensity (specifically the intensity of RLO in the DG) and pathology associated with mortality (metaplasia and/or foot condition) compared to naïve abalone.

During the course of our studies, a phage hyperparasite was observed in black abalone infected with the WS-RLO (phage-infected WS-RLO is named RLOv) and was hypothesized to reduce the intra-host replication of the parasite (Friedman and Crosson, 2012). Therefore, we also predicted that presence of the RLOv would reduce disease in all exposed abalone.

MATERIALS AND METHODS

GENERAL METHODS

Animals

Black abalone ranging in size (defined as the maximum length of the elliptical shell) from 25 to 67 mm were collected from three sites at two study areas to reduce impacts from collections on the populations prior to ESA listing. Abalone were collected from SNI sites 6 (33.215°N, 119.475°W; $n = 10$), 7 (33.219°N, 119.497°W; $n = 16$) and 8 (33.231°N, 119.534°W; $n = 85$) on February 26, 2006 (mean size ± SD = 36.23 ± 8.45 mm; and from the greater Carmel area in central California (Carmel) on February 23–24, 2006 (mean size = 46.22 ± 11.02 mm). Carmel animals were collected from three sites including Granite Canyon (36.436°N, 121.920°W; $n = 37$), Carmel Point (36.544°N, 121.933°W; $n = 37$), and Soberanes Point (36.448°N, 121.929°W; $n = 30$). We received 30 RLO infected red abalone (size range 75–100 mm) from The Abalone Farm, Inc. (35.438°N, 120.894°W; Cayucos, CA, USA) to serve as donor animals to infect black abalone in Trial 1 and used black abalone ($n = 9$ that measured 40–60 mm) from SNI (site 8) as donor animals in Trial 2. In addition, we received 60 red abalone (mean size = ∼30–40 mm) from The Abalone Farm to serve as controls for tank independence in our trials. Following collection in the field all abalone were transported overnight on ice to the School of Aquatic and Fishery Sciences-University of Washington Pathogen Quarantine Facility (UWPQF) and placed in separate recirculating seawater systems at 14°C for an 8-week acclimation period prior to experimental manipulations. Seawater (∼30 psu) was collected from Elliot Bay, Puget Sound, WA, USA and transported to the UWPQF where it was added to each system and maintained at the desired temperature via a heat pump (Delta Star, AquaLogic, Inc., San Diego, CA, USA) and was purified via filtration (25 μm) and ultra violet irradiation followed by an activated charcoal filter. Selected seawater parameters (ammonia, nitrite, and pH) were measured 2× per week prior to weekly partial (∼30%) water changes. Temperature and animal mortality were monitored daily. Animals were fed the algae *Nereocystis luetkeana* (Phaeophyta, Lessoniaceae), *Palmaria mollis* (Rhodophyta, Palmariaceae), and/or *Chondracanthus exasperatus* (Rhodophyta, Gigartinaceae) 2–3× per week. Periodically, feces were collected from each system for assessment of WS-RLO presence via quantitative polymerase chain reaction (qPCR; Friedman et al., 2014). Feces (1–2 ml) were aspirated from each system and the DNA was immediately extracted as outlined below. All effluent from this facility was chlorinated at 10 ppm for 24 h prior to release in the Seattle domestic sewer system as per requirement by the Washington Department of Fish and Wildlife.

Administration and analysis of oxytetracycline

The red abalone (∼30–40 mm group) and all black abalone were medicated with oxytetracycline (OTC) at a dose of ∼90 mg/kg

weight for 3 days according to the methods of Friedman et al. (2007) in order to allow the abalone to purge themselves of RLO infections (Friedman et al., 2003, 2007; Rosenblum et al., 2008). Abalone were maintained in recirculating seawater systems held at 14–15°C for 4 months to allow depletion of OTC to <50 ppm, a level shown to allow re-infection by the WS-RLO (Friedman et al., 2007; Rosenblum et al., 2008). OTC levels in the DG were analyzed according to Association of Official Analytical Chemists [AOAC] (1990) as modified by Friedman et al. (2003, 2007).

Histology

A standard 2–3 mm cross section was excised from each animal sampled to include PE, DG, and foot muscle from all moribund abalone and those sampled at specific time points. Excised tissues were preserved in Davidson's solution (Shaw and Battle, 1957) for 24 h and stored in 70% ethanol until being processed by routine paraffin histology. Deparaffinized 5 μm sections were stained with hematoxylin and eosin (Luna, 1968) and viewed by light microscopy. One to three morphologically distinct RLOs were observed in histological sections and included the WS-RLO, a phage-infected WS-RLO variant (RLOv) and a previously undescribed RLO that is stippled in appearance (ST-RLO; Friedman et al., 1997, 2000; Friedman and Crosson, 2012). The intensities of infections by each RLO type were individually scored according to the following 0–3 histology scale estimating the number of rickettsial colonies per 20× field of view: (0) no infection, (1) 1–10, (2) 11–100, and (3) >100 (Friedman et al., 2002). Tissue changes (metaplasia or foot atrophy) were also scored on the 0–3 scale of Friedman et al. (2002) in which 0 represented normal tissue, 1 indicated <10% change, 2 indicated 11–25% change, and 3 indicated >25% change.

DNA extractions

As the PE is the primary target organ for infection by the WS bacterium (Friedman et al., 2000), this tissue was selected for optimum RLO detection by histology and qPCR, the main diagnostic tools employed in these studies. PE tissue for histology was removed just posterior to the right kidney–DG junction and the next posterior section that contained PE and DG was excised for qPCR. These two sections represent the portion of the gastrointestinal tract where RLO infections are most prevalent (Friedman et al., 2000, 2002, 2007). DNA isolation from PE and DG tissues was performed with a QiaAmp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturers' instructions as modified by Friedman et al. (2007). DNA isolation from feces was performed in the same manner except that initial homogenization was not necessary. All extracted DNA was stored at –20°C until further analysis.

Quantitative PCR

Quantification of WS-RLO DNA was accomplished using the qPCR assay of Friedman et al. (2014). qPCR reactions were conducted using 12.5 μl 2× Immomix (Bioline USA Inc., Taunton, MA, USA), 320 nM of each primer, 200 nM of probe (Biosearch Technologies, Inc., Novato, CA, USA), 0.6 mg/μl BSA, 2 μl of DNA template, and sterile water to bring the final volume to 25 μl per reaction. Thermal cycling conditions included an initial denaturation step of 95°C for 10 min, followed by 41 cycles of 95°C for 15 s, and 60°C for 30 s. Each sample was run in triplicate along with a plasmid-based standard curve of known WS-RLO copy numbers and a negative control. The fluorescence threshold was set at 400 dR for quantification cycle (Cq) determination to allow precise comparison of amplification among reactions. WS-RLO copy numbers were determined for each abalone DNA sample via regression analysis of the standard curve. Gene copy numbers were calculated per gram of sample for tissues and per nanogram of genomic DNA for fecal sample.

EXPERIMENTAL METHODS

Trial 1: Multiple RLO infections: WS-RLO, RLOv, and ST-RLO

After depletion of OTC, abalone from both collection sites (Carmel and SNI) were equally divided among 10 plastic aquaria [12 in × 13.5 in (D × H); Consolidated Plastics, Twinsburg, OH, USA] so that each contained eight abalone and were, in turn, distributed among four 400-l recirculating seawater systems. Two systems were designated as control (no RLO exposure) and two as experimental (RLO exposure) treatments. Each system held two or three tanks from the two black abalone collection sites (SNI and Carmel) for a total of five tanks of black abalone from each population. Each system included a head tank from which water flowed into tanks (**Figure 1**). Abalone were acclimated to the target seawater temperature of 18°C over a 1-week period and maintained at this temperature throughout the study. Seawater systems were maintained and abalone were fed as described above.

One week after reaching 18°C, an equal biomass of red abalone (611 g) was added to each of the four head tanks. RLO-infected red abalone were placed in the head tanks of the experimental systems providing an equivalent dose of the pathogen to each tank, while those free of RLO infection were added to the control systems. After 60 days, red abalone were removed from the head tanks and a sixth tank containing 15 uninfected red abalone was added to each of the four systems (system controls for tank independence).

Each system was checked daily for the presence of moribund (lethargic and weakly attached) animals, which were promptly removed. Selected tissues (PE, DG, and foot muscle) were excised for qPCR and histological analyses. After 162 days, one abalone was removed from each tank and sampled as above. Upon termination of the study, all remaining experimental abalone and two control abalone per tank were sampled as above.

Due to the paucity of abalone available for this study, to better understand host–parasite dynamics, fecal samples were periodically removed from tanks 24 h after feeding and processed for qPCR according to Friedman et al. (2014).

Trial 2: Dual RLO infections (WS-RLO and ST-RLO)

In an effort to compare relative susceptibility of the Carmel and SNI populations without the newly observed RLOv, we repeated the above study using the remaining control abalone and donor abalone from SNI, where the RLOv has not been observed (Friedman and Crosson, 2012). The trial was conducted in the same manner as above with the exception that duplicate tanks holding five animals each were used from each population under the same control and experimental conditions as outlined above. No abalone were sampled during the study except for moribund

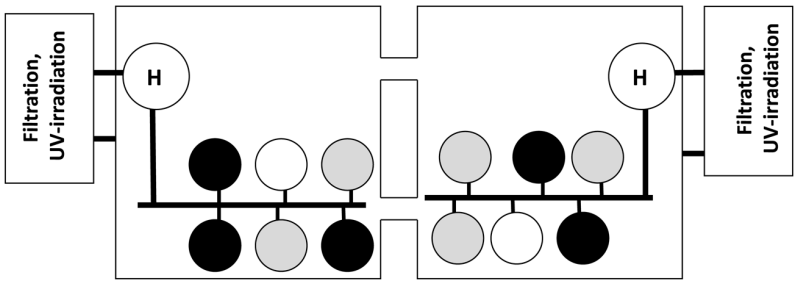

FIGURE 1 | Exposure system used in challenge Trials 1 and 2. Abalone from SNI (black circles) and GC (gray circles) were held in replicate tanks ($n = 5$ in Trial 1 and 2 in Trial 2). The white circles represent tank of uninfected red abalone to test for tank independence in the recirculation system. Seawater flowed from the head tanks (H) holding either infected or uninfected abalone into the exposure tanks. Subsequently seawater flowed into the square sumps and through a series to filters, UV-sterilization and heating systems prior to being returned to the head tank.

or dead individuals. All remaining animals were sacrificed and sampled as above.

Trial 3: Single RLO infections (WS-RLO)

We re-analyzed data from Friedman et al. (2002) in which black abalone from Año Nuevo Island (37.1086°N, 122.3378°W) were exposed to the WS-RLO via cohabitation under the same temperature conditions as in Trials 1 and 2 (18°C) but were held in flowing seawater in lieu of recirculating seawater systems. Abalone used in Trial 3 were larger (mean size = >100 mm) than those used in Trials 1 and 2 (mean size = 49 and 57 mm, respectively). Moribund and surviving abalone were sampled for histology as described above. New analyses of histology and survival data were conducted and are outlined below.

SEA SURFACE TEMPERATURE DATA

Our primary source for sea surface temperature (SST) data at SNI was waverider data buoy 46219 (33.221°N, 119.882°W), moored 32.4 km west of SNI site 8 and operated by the US National Oceanic and Atmospheric Administration (NOAA). Buoy 46219 is also identified as buoy 067 of the Coastal Data Information Program, Scripps Institution of Oceanography, La Jolla, CA, USA. Between September 2004 and December 2012 a total of 42,677 publically available SST measurements were logged hourly, distributed across 46 different months at the buoy site. To verify that data from the buoy site represented SST conditions at SNI, we compared SST data collected in 2007 in the intertidal zone at SNI site 8 with data from the buoy. SNI data were collected between February 1 and December 31 using "TidbiT®" SST loggers (Onset® Computer Corporation, Bourne, MA, USA) cemented to rocky substrata. The data sets included 106 records of paired (within 1 h of one another) SST measurements from the two locations that were logged within 1 h of high tide at SNI. Times of high tides were determined with Nobeltec® Tides and Currents™ software, version 3.5 (Jeppesen Marine, Portland, OR, USA). The requirement for observations at high tide ensured that the data loggers at SNI were fully immersed when SST records were obtained.

STATISTICAL ANALYSES

Histology was used as the primary determinant of infection status. Abalone that were not exposed, but showed signs of infection ($n = 2$ in Trial 2), were discarded from analyses because these animals were most likely contaminated with RLO during the end of the trials. We did not include data from animals that were sampled for histology midway through the trials, but rather used data only from abalone that died or survived until the end of the trials. Our measure of phage infection (RLOv) was either present or absent or ranked density in histological preparations, depending on the analysis. Histological scores for foot condition (atrophy) and RLO density were assumed to be continuous variables on a log scale. Mortality rates of control abalone differed among the trials and this variation in background mortality was controlled for in analyses by nesting the effect of infection within trial. WS-RLO gene copies based on qPCR analyses were included as an additional estimate and acted as a proxy for RLO load (Friedman et al., 2014). To simplify the analyses, we pooled abalone across trials into two groups based on exposure history of disease-selected and naïve. Abalone from SNI were progeny of abalone selected for disease resistance ("selected"), while those from Carmel, where losses due to WS have not been observed (Miner et al., 2006) were considered "naïve." Our survivorship models considered infected (vs uninfected) nested within trial, the interaction between infection status and exposure history, and the interaction between infection status and presence of phage. Here, the interaction terms were the main predictions of our hypotheses, i.e., that the mortality rate of infected abalone relative to control abalone is affected by phage or exposure history to RLOs. We used a proportional hazards model to determine the effect of exposure history and RLOv infection on the relative mortality rates of experimental abalone (for convenience, plots were made with parametric survivorship analysis using a Weibull distribution – results were consistent between the two approaches).

We used logistic stepwise regression to consider a chain of causal mechanisms leading to abalone mortality and pathological indices. For all models, we nested the main effect within trial. Least squares regression was used for all tests except for a nominal regression to contrast mortalities versus survivors. We assumed that all indices were continuous variables due to the presence of several fractional values. Treating these as ordinal variables did not alter the nature of the results. For the linear models, the normality of the residuals was inspected using normal

quantile plots to insure that the assumptions of the test were met. All statistical analyses were run using JMP 10.0 (SAS, Cary, NC, USA).

We calculated Pearson's *r* value to assess the strength of the correlation in SST data between NOAA data buoy 46219 and SNI site 8, using paired data consistent with selection criteria as described above.

RESULTS

SURVIVAL

In the proportional hazards survivorship analysis (78 deaths, 69 survivors), infected abalone had significantly higher mortality rates than control abalone (infection nested in site, $\chi^2 = 29.4$, df = 3, $P < 0.0001$). The relative mortality rate of phage-infected abalone was significantly lower than abalone without phage (interaction between infection and presence of phage, $\chi^2 = 19.0$, df = 1, $P < 0.0001$). There was a near-significant (two-tailed) effect of exposure history (interaction between infection and history of exposure, $\chi^2 = 19.0$, df = 1, $P = 0.066$), with naïve Carmel abalone suffering slightly higher mortality rates than abalone from disease-selected SNI populations, particularly in Trial 1. To illustrate the magnitude of these effects, **Figure 2** shows mortality curves for four categories of infected abalone. Here, the projected effect of phage (RLOv) was to extend the mean (with 95% confidence) time until 50% mortality in infected abalone from 189 (169–211) days (naïve) or 211 (175–251) (selected) days to 399 (352–450) days (naïve) or 443 (386–514) days (selected; **Figure 3**).

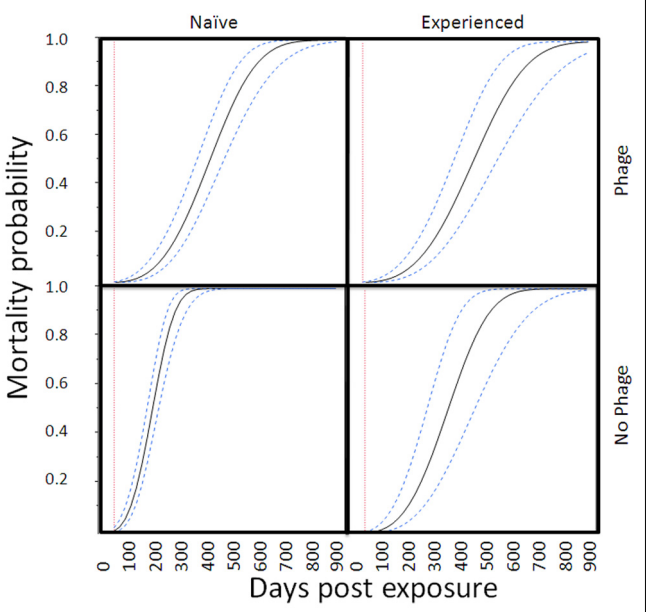

FIGURE 2 | Mortality curves for infected abalone in four categories. The curves (mean values and 95% confidence intervals) are based on a parametric survival fit analysis with Weibull function. For figure simplification, we did not nest infection within trial. This model was consistent with statistics reported from the proportional hazards model.

HISTOLOGY

Abalone mortality significantly increased with pedal atrophy (withered foot) index (nested within trial, $\chi^2 = 65.4$, df = 3, $n = 136$), $P < 0.0001$). Pedal atrophy index significantly increased with DG-RLO index (nested within trial, *F*-ratio = 23.8, df = 3, $n = 133$, $P < 0.0001$). DG-RLO index significantly increased with metaplasia index (nested within trial, *F*-ratio = 67.6, df = 3, $n = 143$, $P < 0.0001$). Metaplasia index significantly increased with PE-RLO index (nested within trial, *F*-ratio = 67.6, df = 3, $n = 143$, $P < 0.0001$). PE-RLO index in infected abalone was not affected by exposure history (nested within trial, *F*-ratio = 0.03, df = 2, $n = 93$, $P = 0.97$). However, in Trial 1, more than half of the PE-RLOs were RLOv (phage-infected WS-RLO), preventing them from contributing to metaplasia and thereby reducing DG-RLO loads as evidenced by reduced DG-RLO indices in these animals (**Table 1**).

Given that exposure history did not influence infection potential based on similar PE-RLO between groups, we conducted a specific test to see if pathological changes due to infection varied between animals that survived and those that died in the three trials. We also examined if exposure history affected other aspects of pathology. Overall, animals that survived had lower levels of metaplasia (index of metaplasia = 0.56) than those that died (index = 1.37; *F*-ratio = 5.5, df = 7, $n = 144$, $P < 0.01$). Within each trial, metaplasia indices of survivors were lower than those that died, with index values of 0.39 and 1.35, respectively, in Trial 1 (*F*-ratio = 6.9, df = 3, $n = 75$, $P < 0.001$), 1.46 and 2.21, respectively, in Trial 2 (*F*-ratio = 1.98, df = 3, $n = 27$, $P = 0.052$), and 0.27 and 0.79, respectively, in Trial 3 (*F*-ratio = 4.5, df = 1, $n = 40$, $P < 0.05$). Presence of the phage (RLOv) reduced both metaplasia (index = 1.12 with phage and 1.68 without phage, *F*-ratio = 4.41, df = 1, $n = 95$, $P = 0.039$) and DG-RLO load (index = 1.38 with phage vs 1.90 without phage, *F*-ratio = 8.99, df = 1, $n = 95$, $P = 0.0035$). The effect of exposure history on infected animals was demonstrated in Trial 1 by the observation of reduced metaplasia upon infection in selected (index = 0.81) relative to naïve animals (index = 1.54; *F*-ratio = 5.30, df = 1, $n = 54$, $P < 0.05$). However, mean metaplasia index was similar between naïve (2.23) and selected (2.00) animals in Trial 2 (*F*-ratio = 0.307, df = 1, $n = 21$, $P = 0.59$; **Table 1**; **Figure 4**). Overall, exposure history affected metaplasia but the effect was not consistent across trials, suggesting, perhaps, that not all the exposed abalone had been under selection for increased resistance at SNI.

qPCR

Naïve infected abalone from Carmel contained ~ 2.5 times more copies of the WS-RLO rDNA gene than did those from disease-selected WS-RLO-infected abalone from SNI [3.82×10^4 (SE = 6.62×10^3) vs 1.47×10^4 (SE = 8.30×10^3) copies per milligram tissue, respectively; *F*-ratio = 4.90, df = 1, $n = 71$, $P = 0.03$]. Abalone that died also contained a mean of 50% more copies of the WS-RLO DNA gene than did survivors. However, these differences were not significantly different from one another [4.28×10^4 (SE = 6.85×10^3) vs 1.28×10^4 (SE = $8. \times 10^3$) copies per milligram tissue, respectively; *F*-ratio = 2.10, df = 3, $n = 71$, $P = 0.22$]. Although infected Carmel abalone excreted 12 times more copies of RLO DNA per nanogram fecal DNA than

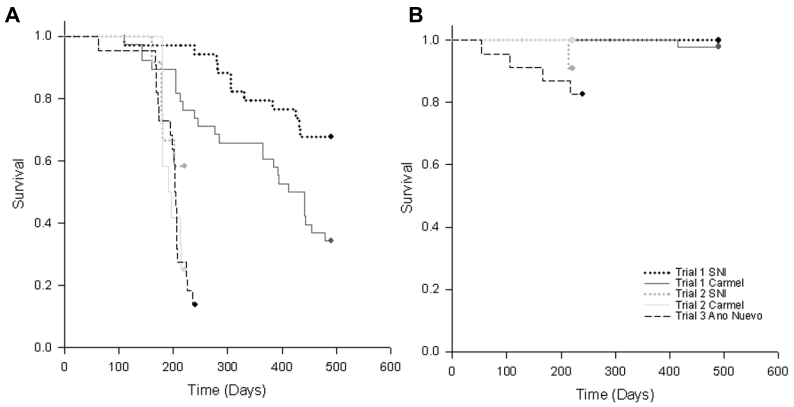

FIGURE 3 | Kaplan–Meier survivorship curves for (A) RLO-exposed and (B) control (unexposed) black abalone from Trials 1–3 in which abalone from Carmel and San Nicholas Island (SNI) were exposed to three RLOs (WS-RLO, ST-RLO, and RLOv) in Trial 1, two RLOs (WS-RLO and ST-RLO) in Trial 2, or one RLO (WS-RLO) in Trial 3. Survival trends for abalone from the three collection sites are represented by dotted lines (SNI), solid lines (Carmel) and dashed lines (Año Nuevo Island).

Table 1 | Histology trends among groups sampled as mortalities relative to those that survived within each trial.

Trial	Site	Mortality or survivor	No.	Mean foot	Mean metaplasia	PE-RLO	PE-ST	PE-RLOv	DG-RLO	DG-ST	DG-RLOv	Mean total PE-RLOs	Mean total DG-RLOs
1	SNI[1]	M	12	**1.6**	*1.2*	1.5	0.6	1.8	*0.9*	0.4	0.8	2.0	1.1
	SNI	S	10	**0.1**	*0.3*	1.7	0.4	1.9	*0.4*	0.1	0.6	2.1	0.6
	Carmel	M	24	**2.0**	1.5	1.7	0.8	1.7	1.3	0.8	*1.0*	2.3	1.6
	Carmel	S	10	**0.5**	1.3	1.7	0.5	1.9	1.5	0.4	*1.5*	2.2	1.7
2	SNI	M	5	**2.1**	2.0	2.6	0.6	0.0	1.9	0.0	0.0	2.6	1.9
	SNI	S	4	**0.6**	1.7	2.3	0.6	0.0	1.5	0.5	0.0	2.3	1.7
	Carmel	M	10	2.7	2.3	2.3	1.8	0.0	**2.5**	0.6	0.0	2.4	**2.5**
	Carmel	S	2	2.1	1.7	1.3	1.3	0.0	**1.5**	1.3	0.0	1.8	**1.7**
3	Año[2]	M	21	1.5	*1.0*	2.3	0.0	0.0	1.5	0.0	0.0	2.3	1.5
	Año	S	3	2.0	*2.0*	2.7	0.0	0.0	2.3	0.0	0.0	2.7	2.3
1, 2, 3	Controls	S	39	0.1	0.1	0	0	0	0	0	0	0	0

RLO infection intensities were scored by estimating the number of rickettsial colonies per 20× field of view: (0) no infection, (1) 1–10, (2) 11–100, and (3) > 100 (Friedman et al., 2002). Tissue changes (metaplasia or foot atrophy) were similarly scored: 0 represented normal tissue, 1 indicated < 10% change, 2 indicated 11–25% change, and 3 indicated > 25% change. Bold indicates significant differences in measured response of exposed abalone between sampling periods (mortality or survivor) within a collection site. Italics indicate nearly significant differences between mortalities and survivors within a collection site (P = 0.052–0.069). Underlined data indicate significant differences in measured response between abalone with different exposure histories (collection site) and whether they were animals that died (mortality, M) or survived (S) to be sampled. [1] *San Nicolas Island.* [2] *Año Nuevo Island.*

did infected abalone from SNI [1.9×10^4 (SE = 9.73×10^3) vs 0.16×10^4 (SE = 9.73×10^3) copies per nanogram genomic DNA], these differences were not significant (F-ratio = 1.60, df = 1, $n = 17$, $P = 0.22$).

SEA SURFACE TEMPERATURE

We found that 17.7% of SST records from NOAA buoy 46219 were ≥17°C and 5.3% ≥18°C (**Table 2**). During the period of record many events of ≥4 h duration occurred in which 17.0 ≤ SST < 18.0°C, and with SST > 18.0°C (**Table 2**). The correlation between paired SST records from the buoy and from SST loggers at SNI site 8 was significant (Pearson's $r = 0.71$, df = 104, $P < 0.001$).

DISCUSSION

Our findings suggest that the increased survivorship seen at SNI was due to reduced metaplasia and WS-RLO loads in the DG. While we found some evidence for our hypothesis of the evolution of resistance in black abalone following mass mortality, the significance of these results varied among trials. It is possible that selection for resistance varied among the samples we used in our experiments (which were taken at different times). Furthermore, sample sizes were small in Trial 2, which combined with a shorter trial duration may have influenced our ability to detect differences in measured metrics between groups. Trial 2 (lacking phage) was terminated after day 85 (when no abalone deaths had occurred for 2 weeks). Although the slopes of the mortality curves

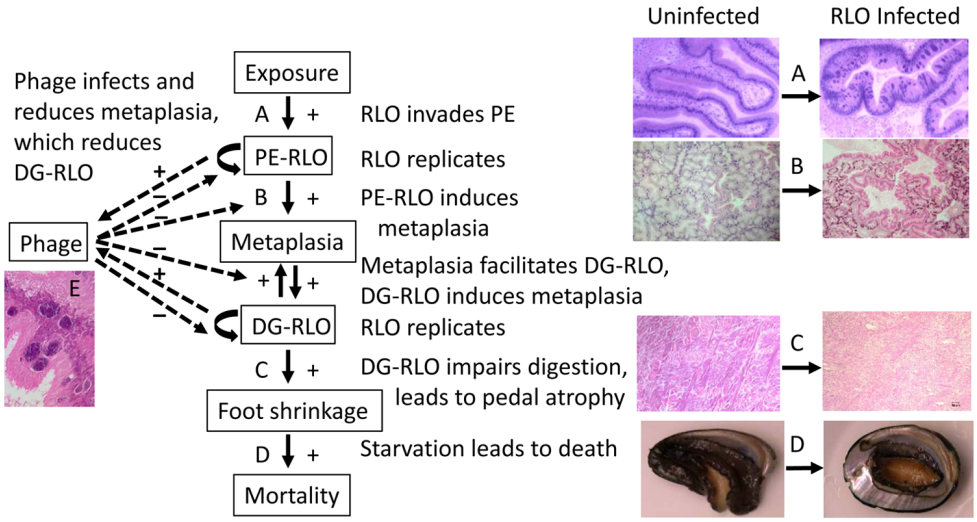

FIGURE 4 | Flow chart illustrating the relationship among abalone host, WS-RLO parasite, and phage hyperparasite of WS-RLO. Arrows indicate the flow of the effect with an increase in the response denoted with a "+" and a reduction in the response shown by a "−". WS-RLO exposure results in infection of the posterior esophagus (PE) **(A)**, which leads to metaplasia in the digestive gland (DG) **(B)**. Metaplasia, in turn, provides more target tissue for the WS-RLO to invade and infect this organ, which together result in dysfunction of the DG and catabolism of the pedal muscle as an energy source **(C)**. Visible atrophy of the pedal muscle becomes apparent in the withered and lethargic abalone at the end-stage of disease **(D)**. Phage infection of the WS-RLO (RLOv) reduces the amount of active WS-RLO, thereby reducing metaplasia and DG-RLO, which in turn reduces pedal atrophy and mortality **(E)**. Magnifications: **(A)** 100×, **(B,C)** 40×, **(D)** 1×, **(E)** 400×.

Table 2 | Frequency and duration of occurrence of sea surface temperatures (SSTs) sufficiently high to increase the risk of black abalone to effects of withering syndrome at San Nicolas Island (SNI), California USA.

	SST ≥ 17°C	SST ≥ 18°C	17 ≤ SST < 18°C	SST ≥ 18°C
Frequency (n = 42,677 hourly observations)	7,559 (17.7%)	2,253 (5.3%)	–	–
Number of episodes	–	–	200	73
Mean duration (range)	–	–	14.1 h (0.5–718 h)	15.9 h (0.5–257 h)

Data are publically available and were collected hourly by NOAA waverider buoy 46219, located 32.4 km west of black abalone study site 8 at SNI. Data collection was intermittent from September 2004 through December 2012. Minimum episode duration was set at 4 h. Data from the buoy were significantly correlated with temporally paired SST data obtained during high tide in black abalone habitat at site 8 in 2007.

for exposed animals in Trial 2 were similar, ∼2× more SNI abalone survived than those from Carmel (**Figure 3A**). Thus, had the study continued longer, we might have seen differences in survival between exposure history arise as seen in trail one. Despite inferential constraints imposed by sample size limitation, differences in mortality of naïve relative to disease-selected abalone when exposed to the WS-RLO were consistent with effects observed in Trial 1.

The ability for marine mollusks to evolve disease resistance has been investigated in a number of species with varying results. A viral disease of abalone (abalone viral ganglioneuritis, AVG) in Australia caused high mortality along the coast of Victoria in 2005–2006 and surviving abalone tested 5–6 years later remained susceptible to the virus suggesting a lack of evolved resistance (Crane et al., 2013). The ephemeral nature of AVG and possibly insufficient time for significant recruitment post-selection may explain the apparent lack of developed resistance to this disease. The development of disease resistance has been investigated in oysters infected with protistan and viral pathogens. After four generations of selection beginning with American oyster *Crassostrea virginica* broodstock that had survived disease pressure from two protistan pathogens for 2–5 years, cumulative survival more than doubled when exposed to the protists (Calvo et al., 2003) illustrating the development of resistance over time. Resistance in populations has also been observed in Pacific oysters *Crassostrea gigas* after several generations of selection to a herpes virus (ostreid herpesvirus-1); heritability of traits was also high (e.g., h^2 = 0.61–0.95; Dégremont et al., 2010). These examples illustrate a genetic basis for resistance in marine mollusks and that rates of selection for resistance vary among species. The black abalone from SNI were likely first or second generation progeny of those that survived the initial losses of ≥95% due to WS given their observation 10 years after the first observation of WS on SNI in 1992 and ∼5–6 years after ∼90% mortality (VanBlaricom et al., 1993; Crosson et al., 2014). It is possible that abalone have limited ability to evolve resistance to novel pathogens like AVG and WS-RLO; however our data suggest that over time enhanced resistance to the

WS-RLO is likely to increase in populations experiencing disease pressure.

Given the known association of WS-induced mortality rates with warm-water events such as ENSO episodes, it is plausible that the recently observed global-scale hiatus in sea surface warming (e.g., Meehl et al., 2011; Kosaka and Xie, 2013) may have mitigated detrimental effects of WS on black abalone populations in southern California. However, we are skeptical that ocean temperature trends alone can explain observed positive trends in black abalone population densities at SNI and elsewhere in the southern California Islands for two reasons. First, our evaluation of relevant SST data indicated that periods of SST high enough to enhance vulnerability of black abalone to WS at SNI were frequent and often of substantial duration (continuous for up to 30 days), despite the hiatus in ocean warming. Second, we would have expected a regional climate signal to have led to signs of incipient population-scale recovery at more than just a small number of sites.

Our finding that the phage effectively eliminates the ability of infected WS-RLOs to cause disease was unexpected. Seasonal losses due to WS in California farms have lessened over the past several years since the phage was observed in farmed animals suggesting that natural phage-therapy operates under farm conditions (R. Fields, personal communication), and this effect may also apply to the field. However, whether or not phage commonly infects wild abalone remains to be seen.

Viroplankton (especially bacteriophages) are the most abundant biological elements in the marine environment and play key roles in driving host population dynamics (see review by Suttle, 2005). The observation of a phage hyperparasite in the WS-RLO is therefore not surprising. A number of phages have been reported infecting a variety of marine bacteria including RLOs (see detailed list in Friedman and Crosson, 2012). Hyperparasites have been shown to influence primary host–parasite relationships and recent renewed interest in phage therapy has highlighted the importance of the interplay among host immune response, primary pathogen and hyperparasite (Sabelis et al., 2002). Given the intracellular nature of the RLOs and their location in the digestive epithelium, the host immune response may play less of a role in WS than in bacterial septicemia. Infection of a prokaryote by a phage results in death of the host cell by lysis (lytic cycle; see reviews by Young, 1992, 2002) or by induced changes in physiology through co-opting of the host cell to produce more phages, and by lysogeny (Kourilsky, 1973; Weinbauer, 2004; Bobay et al., 2013). Lytic phages have been used as a therapeutic alternative to antibiotics in many systems including those of bacterial pathogens in marine species. Bacteriophages were recently reported as an alternative control for *Vibrio anguillarum* infecting Atlantic salmon (Higuera et al., 2013) and *V. parahaemolyticus* infections in brine shrimp (Martínez-Díaz and Hipólito-Morales, 2013). In both cases, efficacy of the phage therapy was based on phage-induced lysis of its bacterial host. Some phages integrate into the host genome (lysogenize) and are replicated and passed on to the next generation via host cell division (Kourilsky, 1973; Golais et al., 2013). Lysogeny has been shown to increase the virulence and pathogenicity of bacterial pathogens including those infecting marine species such as *V. harveyi* in shrimp (Ruangpan et al., 1999; Khemayan et al., 2006). Shrimp infected with phage-lysogenized *V. harveyi* experienced over 100 times the death rate of those lacking infection with the phage *V. harveyi* siphophage 1 (VHS1; Intaraprasong et al., 2009).

The phage infecting the WS-RLO appears unique in its ability to alter the course of infection, not by lysis of its bacterial host but apparently by effectively eliminating normal function of the WS-RLO. This is evidenced by a reduction in the effect of PE infection on the DG and resulting reduced mortality of the abalone host and functioning as a natural phage therapy. Approximately half of the WS-RLO colonies were phage infected (RLOv), thereby effectively reducing the pathogenic infection load by half. Increased host tolerance to WS-RLO infection in the PE may also contribute to the observed reduction in metaplasia and DG-RLO loads. Lysis of RLOv cells has not been observed by electron microscopic examination (Friedman and Crosson, 2012), but presumably occurs at some level in order to effect phage transmission. Alternatively, after its initial infection of the WS-RLO, transmission of the phage may rely primarily on lysis of the host abalone gastrointestinal epithelial cells by the RLOv followed by infection of new host cells and or hosts. Lysis of WS-RLO infected gastrointestinal cells has been observed by electron microscopy (Moore and Friedman, unpublished data). A lack of RLOv lysis suggests that the phage may be defective or that signals to induce host cell lysis are rare (Young, 1992, 2002; Golais et al., 2013).

CONCLUDING REMARKS

It is encouraging that black abalone are surviving longer and recruiting to SNI. This does not seem to be fully explainable by stabilized SSTs in years since the original mass mortality. While our results suggest that some abalone at SNI have evolved increased resistance to the RLO, evidence for an evolved response was not strong. Differences in survival between exposure histories and tolerance to infection were enhanced with phage presence. The phage seen in our experiments substantially increased the longevity of infected abalone and, if prevalent in the wild, might help this endangered species recover from the brink of extinction.

AUTHOR CONTRIBUTIONS

All authors helped write and edit the manuscript with Carolyn S. Friedman as the primary author. Carolyn S. Friedman also designed the experiments, read slides, analyzed data, made and or edited tables/figures. Kevin D. Lafferty analyzed data, created **Figure 2**, and designed **Figure 3**. Nathan Wight and Lisa M. Crosson conducted Trials 1 and 2, extracted DNA, sampled animals, and conducted qPCR assays. Glenn R. VanBlaricom collected abalone for experiments, conducted temperature analyses, and created **Table 2**.

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Mycoloop: chytrids in aquatic food webs

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Parasites are ecologically significant in various ecosystems through their role in shaping food web structure, facilitating energy transfer, and controlling disease. Here in this review, we mainly focus on parasitic chytrids, the dominant parasites in aquatic ecosystems, and explain their roles in aquatic food webs, particularly as prey for zooplankton. Chytrids have a free-living zoosporic stage, during which they actively search for new hosts. Zoospores are excellent food for zooplankton in terms of size, shape, and nutritional quality. In the field, densities of chytrids can be high, ranging from 10^1 to 10^9 spores L^{-1}. When large inedible phytoplankton species are infected by chytrids, nutrients within host cells are transferred to zooplankton via the zoospores of parasitic chytrids. This new pathway, the "mycoloop," may play an important role in shaping aquatic ecosystems, by altering sinking fluxes or determining system stability. The grazing of zoospores by zooplankton may also suppress outbreaks of parasitic chytrids. A food web model demonstrated that the contribution of the mycoloop to zooplankton production increased with nutrient availability and was also dependent on the stability of the system. Further studies with advanced molecular tools are likely to discover greater chytrid diversity and evidence of additional mycoloops in lakes and oceans.

Keywords: parasitic fungi, chytridiomycota, diatom, daphnia, mycoloop, indirect mutualism, stability, trophic transfer

ECOLOGICAL SIGNIFICANCE OF PARASITES

Parasites are important components of ecological communities (Thomas et al., 2005; Hatcher and Dunn, 2011). They have the potential to regulate host populations, mediate interspecific competition between hosts and other species, maintain genetic polymorphism and biodiversity, and affect community structure. Nevertheless, the effects of parasites and diseases on food webs and ecosystem dynamics have been neglected until recently (Polis and Strong, 1996; Marcogliese and Cone, 1997). New research suggests that parasites have the potential to alter food-web topology, stability, interaction strength and energy flow (Lafferty, 2006; Kuris et al., 2008; Lafferty et al., 2008).

Parasites commonly function as prey within ecosystems (Johnson et al., 2010; Thieltges et al., 2013). There are two main ways in which parasites become prey. Predators can either consume the infected hosts of parasites (concomitant predation) or their free-swimming life stage (Johnson et al., 2010). Many aquatic parasites including viruses, chytrids, trematodes, and nematodes, have a free-swimming stage that may be subject to predation (Gonzalez and Suttle, 1993; Kagami et al., 2004; Kuris et al., 2008; Johnson et al., 2010). The Chytridiomycota (chytrids) are one of the dominant groups of parasites in aquatic ecosystems. The free-living zoosporic stage of chytrids actively searches for and infects host cells, extracting nutrients and developing into mature sporangia that release new zoospores (Canter, 1967; **Figure 1**). There are more than 700 species of chytrids known to infect phytoplankton, zooplankton, fungi, plants, and invertebrate animals (Sparrow, 1960; Gleason et al., 2008). Here in this review, we mainly focus on parasitic chytrids that infect phytoplankton, and explain their roles in aquatic food webs as prey for zooplankton through the "mycoloop" pathway (Kagami et al., 2007a).

CHYTRIDS ZOOSPORES AS PREY FOR ZOOPLANKTON: FOOD QUALITY AND QUANTITY, AND THE MYCOLOOP

Predation of parasites can be beneficial to predators if they can gain energy and nutrition from parasites. Chytrid zoospores are a good food source for zooplankton in terms of size and shape (Kagami et al., 2004). In addition, zoospores are rich in polyunsaturated fatty acids (PUFAs) and cholesterol, which are essential for the growth of crustaceans (Kagami et al., 2007b). Zooplankton, such as cladocerans (*Daphnia*) and copepods, are able to grow by acquiring important supplementary nutrients from a diet of zoospores (Kagami et al., 2007b, 2011). Thus, chytrids may improve zooplankton production and enhance trophic transfer.

Many recent studies suggest that parasite biomass is not negligible, and may in fact be often significantly high (Kuris et al., 2008). The abundance of chytrids in aquatic systems has been found to be much higher than traditionally thought. Because morphological identification of chytrid zoospores is difficult, attempts have been made to use the fluorescent stains to count the density of chytrid zoospores in lakes (Kudoh, 1990). Recently, molecular techniques, such as CARD-FISH (Jobard et al., 2010) and Real-Time qPCR (Lefèvre et al., 2010) have been applied to estimate zoospore abundance in field samples and have detected zoospore concentrations of 10^1–10^6 spores L^{-1} (**Table 1**).

Compared to quantifying the abundance of zoospores, sporangia are much easier to count because they are attached to host phytoplankton cells. Using prevalence of infection and host cell

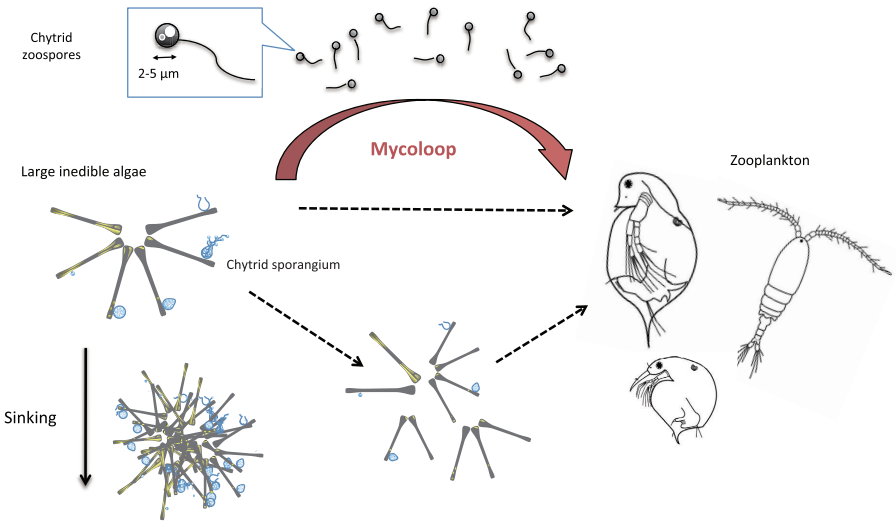

FIGURE 1 | Diagram of "mycoloop." Parasitic chytrids can transfer material from large inedible phytoplankton to zooplankton. Chytrids zoospores are excellent food for zooplankton in terms of size (2–5 µm in diameter), shape, nutritional quality (rich in PUFAs and cholesterols). Large colonies of host phytoplankton may also be fragmented by chytrid infections and become edible to zooplankton. On the other hand, infected host colonies may remain inedible to *Daphnia*, or even become less edible due to the aggregate formation of cells. Those aggregations may sink faster, and affect material cycling in lakes.

density data from the literature, we determined the density of sporangia to be 10^1–10^8 spores L^{-1} in field surveys (**Table 1**). The result indicated that direct counts of zoospores in the field may underestimate the real densities. By using the zoospore per sporangia conversion factors determined by previous studies (13–28 zoospores per sporangium, Sen, 1988; 4–25 zoospores per sporangium, Bruning, 1991), or the zoospore per sporangium biovolume conversion (0.166 per μm^3 empty sporangium volume, Bruning, 1991), we can roughly estimate that zoospore abundance could actually reach more than 10^9 zoospores L^{-1} in the field. It should be noted that lowest abundance of zoospore can be 10 spores L^{-1}, or even zero. This indicates that the potential importance of mycoloop may vary with seasons and lakes.

Molecular studies have also revealed that chytrid zoospore may often be miscounted as small heterotrophic nano-flagellates (HNF), due to similar forms and sizes (Sime-Ngando et al., 2011). A significant portion of small eukaryotes (0.6–5 µm) was recently found to be chytrid zoospores in freshwater lakes (11–23%, Lefèvre et al., 2007; 30% Lepère et al., 2008). In addition, CARD-FISH identified 5–60% of unknown flagellates as chytrids zoospores (Jobard et al., 2010). In aquatic ecosystems, most small heterotrophic eukaryotes (<5 µm) are considered to play a role in microbial food webs by acting as predators of bacteria and bacterium-sized phytoplankton (Sherr and Sherr, 1983). In contrast, chytrids consume phytoplankton directly as parasites, and they do not eat bacteria. These findings require that we should revise our understanding of microbial food webs.

Zoospores may become particularly important to *Daphnia* when large inedible phytoplankton species, such as the diatom *Asterionella*, dominate the phytoplankton community. Large phytoplankton species are quite resistant to grazing by zooplankton such as *Daphnia* (Knisely and Geller, 1986; Kagami et al., 2002). However, if large inedible phytoplankton species are infected by chytrids, then nutrients within host cells are consumed by chytrids and can be grazed by *Daphnia*. This new pathway has been dubbed the "mycoloop" since nutrients from large inedible algae are transferred to zooplankton via the zoospores of parasitic chytrids (Kagami et al., 2007a).

Trophic transfer efficiency from host algae to chytrids is an essential parameter to examine the importance of mycoloop in the field. The transfer efficiencies of carbon, nitrogen, and phosphorus (CNP) from host *Asterionella* populations to free-swimming zoospores were estimated to be 6–9% in the laboratory experiment, when the prevalence of infection was about 60% (Kagami et al., 2007b). Those efficiencies were population based, and may become even higher if the prevalence of infection may exceed 90%. While, a single zoospore can use the host tissues quite efficiently because chytrid can directly consume host nutrients by entering through a germ tube (Van Donk and Ringelberg, 1983). CNP concentrations in a single zoospore (10.7 pg C, 0.6 pg N, 2.4 pg P per zoospore, Kagami et al., 2007b) are comparable to 20% of those in single host cell, indicating just five zoospores may be enough to exploit all algal tissues. 20% must be overestimated, if we consider the range of zoospores per sporangia (4–25 zoospores per sporangium, Bruning, 1991). We need to measure the CNP concentrations of zoospores and host cells, and number of zoospores per sporangium accurately with different species and conditions. From these estimates, we can examine how important chytrid zoospores may be as a food source for zooplankton in the field, in comparison to other possible food sources. In addition, such estimates are also crucial for modeling approaches, to predict the roles of chytrids in altering the network structure and stability (Niquil et al., 2011), and in determining the zooplankton production (Miki et al., 2011).

Table 1 | Density of zoospores or sporangia of chytrids in lakes.

Zoospores (10^3 L^{-1})		Sporangia (10^3 L^{-1})		Methods	Chytrid	Host	Lakes (Trophic status)	References
min	max	min	max					
1	360			Count with Nile Red and DAPI	*Rhizophydium, Zygorhizidium*	*Asterionella Formosa*	Lake Suwa, Japan (E)	Kudoh, 1990
89 ± 11	156 ± 51			CARD-FISH (<25 um)	Chytridiales (*Rhizophidium, Chytridium**)	*Melosira, Anabaena*	Lake Aydat, France (E)	Jobard et al., 2010
52 ± 11	573 ± 68			CARD-FISH (<25 um)	Chytridiales (*Rhizophidium, Chytridium**)	Various species[a]	Lake Pavin, France (OM)	Jobard et al., 2010
0.04	5			qPCR	Rhizophidiales (parasitic and saprotrophic)	Unknown	Lake Pavin, France (OM)	Lefèvre et al., 2010
0.019	0.454			qPCR	*Badrachochytrium dendrobatidis*	Amphibians	Lakes and ponds, USA	Kirshtein et al., 2007
			1085	Direct count (Utermöhl)	*Zygorhizidium. planktonicum*	*Asterionella Formosa*	Lake Maarsseveen, The Netherlands (OM)	Van Donk and Ringelberg, 1983
			510	Direct count (Utermöhl)	*Zygorhizidium affluens*	*Asterionella Formosa*	Crose Mere, UK	Reynolds, 1973
		1	562	Count with CFW	*Zygorhizidium**, *Chytridium**	*Aulacoseira granulata, A. ambigua*	Lake Inba, Japan (E)	Kagami et al., 2012
		0.524	368	Count with CFW	*Rhizophidium, Chytridium, Zygorizihidium*	Various species[b]	Lake Pavin, France (OM)	Rasconi et al., 2012
			31500	Count with CFW	*Rhizophidium, Chytridium, Zygorizihidium*	Various species[c]	Lake Aydat, France (E)	Rasconi et al., 2012
		1	120	Host density × %	*Rhizophydium, Zygorhizidium*	*Asterionella Formosa*	Lake Suwa, Japan (E)	Kudoh and Takahashi, 1990
		0.4	65	Host density × %	*Rhizophydium**	*Staurastrum dorsidentiferum*	Lake Biwa, Japan (M)	Kagami and Urabe, 2002
			40	Host density × %	*Rhizidium microcystidis*	*Microcystis aeruginosa*	Shearwater, UK (E)	Sen, 1988
		5	1486	Host density × %	*Rhizophidium planktonicum*	*Asterionella Formosa*	Lake District, UK (OM)	Canter and Lund, 1953
		6	110	Host density × %	*Rhizophidium flagilariae, Chytridium versatile*	*Fragilaria crotonensis*	Lake District, UK (OM)	Canter and Lund, 1953
		0.04	1	Host density × %	*Rhizophydium couchii*	*Staurastrum* spp.	Lake District, UK (OM)	Canter and Lund, 1969
			1500	Host density × %	*Zygorhizidium* sp.	*Stephanodiscus parvus*	Lake Schohsee, Germany (M)	Holfeld, 1998
			170	Host density × %	Unknown monocentric chytrid	*Chrysamoeba radians*		
			660	Host density × %	*Rhizophydium planktonicum, R. tetragenum, Zygorhizidium planktonicum*	*Asterionella formosa*		

(Continued)

Table 1 | Continued

Zoospores (10^3 L^{-1})		Sporangia (10^3 L^{-1})		Methods	Chytrid	Host	Lakes (Trophic status)	References
min	max	min	max					
			40	Host density × %	*Zygorhizidium* sp.	*Fragilaria crotonensis*		
			9	Host density × %	*Zygorhizidium. planktonicum*	*Synedra acus*		
			30	Host density × %	*Haparopera piriformis*	*Ankyra judayi*		
			10	Host density × %	*Zygorhizidium parallelosede*	*Elakatothrix genevensis*		

Minimum densities during the presence of chytrids were shown if available. The density of sporangia was estimated from the prevalence of infection and host cell density data from the literature (shown as "Host density × %").

CFW, calcofluor white; OM, oligo-mesotrophic; M, mesotrophic; E, eutrophic.

**Uncertain identification based on morphology or phylogeny.*

[a]Asterionella, Fragilaria, Synedra, Staurastrum, Oocystis.

[b]Asterionella, Synedra, Staurastrum, Cyclotella, Fragilaria, Ankira, Melosira, Starodesmus, Chodatella, Ankystrodesms, Cylindrospermum, Oocystis.

[c]Asterionella, Synedra, Staurastrum, Cyclotella, Fragilaria, Ankira, Melosira, Oscillatoria, Microcystis, Fragilaria, Gomphosphaeria, Anabaena.

The "mycoloop" may occasionally play an important role in shaping aquatic systems, by altering the material flow (**Figure 1**). Traditionally, large inedible phytoplankton species are believed to be lost by sinking from the euphotic zone (Malone, 1981). However, if large phytoplankton are parasitized, then nutrients within host cells are instead consumed by chytrids, and can, in turn, be grazed by *Daphnia* through the mycoloop (Kagami et al., 2007a). In addition, large inedible colonies of phytoplankton may be fragmented into smaller pieces due to chytrid infections, making them more edible to zooplankton (**Figure 1**, Sime-Ngando, 2012). The trophic transfer efficiency from large phytoplankton to *Daphnia* would not change largely even if the heavily infected colonies are fragmented and grazed (i.e., after most of the host cells are consumed by chytrid). It would change, however, if the lightly infected host colonies are fragmented and grazed (i.e., before most of the host cells are consumed by chytrid) (Sime-Ngando, 2012). In this way, nutrients in host phytoplankton cells are partly incorporated into the food web in the euphotic zone, instead of sinking.

On the other hand, some of the infected host colonies may remain inedible for *Daphnia*, or even become less edible due to the aggregation of cells (Kagami et al., 2005, **Figure 1**) and may sink faster than single colonies. In addition, frustules of previously infected cells may sink faster than living cells (Kagami et al., 2006). In this way, sinking of frustules and aggregates of host cells may actually be facilitated by chytrid infections.

PREDATION ON CHYTRIDS MAY SUPPRESS OUTBREAKS OF CHYTRIDS

Predation on the free-living stages of parasites may result in reduced disease risk for hosts (Packer et al., 2003). Indeed, the presence of *Daphnia* can decrease chytrid infection intensity on phytoplankton (Kagami et al., 2004). Recent studies also revealed that *Daphnia* grazing on free-swimming zoospores of *Batrachochytrium dendrobatidis* can decrease the disease chytridiomycosis of amphibians (Hamilton et al., 2012; Searle et al., 2013).

PHYTOPLANKTON—CHYTRID—ZOOPLANKTON INTERACTIONS

Although a short-term experiment demonstrated that the direct trophic link from chytrid fungus to zooplankton (F-Z link) increased zooplankton growth (Kagami et al., 2007b), the effects of the F-Z link on food web dynamics is not easily predictable.

Considering that chytrid infections are common in large inedible phytoplankton species (Sommer, 1987; Kagami et al., 2007a), fungal parasitism may indirectly increase the abundance of small edible phytoplankton by altering resource competition. This may in turn enhance zooplankton production through grazing pathways via an "indirect mutualism" (Levine, 1976; Vandermeer, 1980). If the F-Z link then decreases the abundance of fungal zoospores (or fungal parasitism), it weakens the indirect mutualism between fungi and zooplankton, and will then decrease material transfer from small phytoplankton to zooplankton (indirect effect).

Therefore, the F-Z link may enhance zooplankton production through the mycoloop (direct effects), while it may also decrease zooplankton production by weakening indirect mutualism (indirect effect). By using a simple food web model, we successfully evaluated the effects of parasitic chytrids (fungal parasitism) and the F-Z link (both direct and indirect effects) on food web dynamics (Miki et al., 2011). In summary, presence of the F-Z link caused unexpected indirect effects in the food web, and was an important determinant for the stability of the system (see the following section for more detail). The model indicated that the high growth efficiency and high nutritional quality of fungi were crucial for the F-Z link to increase zooplankton production. The model also indicated that the contribution of the mycoloop (material transfer via the F-Z link) to zooplankton production increased with nutrient availability and depended on the system stability. This

implies that neglecting the dynamical aspect of the system will lead to inaccurate estimates of material and energy fluxes. In the following section, we will review the theoretical approaches in detail to evaluate the roles of parasitic fungi in aquatic food webs.

MULTIFACETED IMPACTS OF FUNGUS-ZOOPLANKTON INTERACTIONS ON FOOD WEB DYNAMICS: LESSONS FROM DYNAMICAL MODELS

There are two modeling approaches for describing the structure, dynamics, and fluxes of material and energy in food webs and ecosystems: steady state models and dynamical models. The steady state model (or linear model) is a powerful tool in ecosystem sciences to quantitatively estimate material fluxes with limited observations (Vezina, 1989). For example, it has been used to estimate the impacts on carbon fluxes in aquatic ecosystems, of the microbial loop (e.g., Anderson and Ducklow, 2001; Anderson and Tang, 2010), bacteriophage (Fuhrman, 1999; Motegi et al., 2009), and the food web structure (Niquil et al., 2006). Recent studies also quantified the impact of chytrid fungi in lake carbon fluxes using this modeling approach with inverse estimates of fluxes (Grami et al., 2011; Niquil et al., 2011). On the other hand, the dynamical model, which often requires a larger number of parameters and more specific mathematical formulations for inter-compartment interactions (e.g., trophic and competitive interactions), can provide information about both the steady state structure and non-steady state dynamics of the food web. Here, we would like to highlight the three major impacts of the F-Z link on the food web; (1) effect on food web structure and zooplankton production, (2) influence on system stability, and (3) contribution to material fluxes (mycoloop), elucidated by dynamical food web models (Miki et al., 2011; Gerla et al., 2013). Since conclusions are often different between steady state models and dynamic models, we will compare these two modeling approaches.

1. *Effects of the F-Z link on food web structure and zooplankton production:* The dynamical model can predict the unexpected consequences of nonlinear effects of adding or removing a specific trophic linkage or a specific player in the structure of the food web (Pimm, 1991). In our case, we added/removed two trophic linkages; fungal parasitism and the F-Z link (Miki et al., 2011) for a detailed comparison among three scenarios: the system without parasitic fungi, the system with fungal parasitism but without the F-Z link, and the system with both fungal parasitism and the F-Z link. The dynamical model predicts that the F-Z link indirectly lowers the abundance of small phytoplankton, altering the food web structure (Miki et al., 2011). This prediction agrees with the predicted decline in picophytoplankton production in Lake Pavin steady state model (Grami et al., 2011). Similarly, both models predict the positive impact of fungal parasitism and F-Z link on zooplankton production and biomass. This implies robust positive impacts of fungi on trophic transfer to higher trophic levels. Some scenarios in the dynamical model have not been explored in the framework of the steady state model. The dynamical model predicted that the F-Z link can unexpectedly reduce the production and biomass of zooplankton compared to a system with fungal parasitism but without an active F-Z link (Miki et al., 2011). This is unexpected considering the apparent (direct) benefit of the F-Z link to zooplankton. This occurs through an indirect effect; the F-Z link increases the host population (large inedible phytoplankton) via a top-down cascade, which in turn decreases the population of non-host, small edible phytoplankton through intensified resource competition (**Figure 2**). In particular, when the growth efficiency of parasitic fungi on host tissues and the nutrient quality of zoospores for zooplankton are not large enough (i.e., the metabolic loss through these trophic interactions is large) or productivity (nutrient availability) in the system is low, then indirect negative effects are greater than positive direct benefits and the F-Z link then causes a reduction in the zooplankton biomass and production (compare A vs. B in **Figure 2**). In order to better quantify the role of fungal parasitism and the F-Z link separately, three scenarios (the system without fungi, the system with fungal parasitism but without trophic transfer from fungi to zooplankton, and the system with both fungal parasitism and F-Z link) should be compared even with the steady state model approach.
2. *Influence of the F-Z link on system stability:* Although theoretical metrics for steady-state ecosystem structure can predict the complexity of the network and imply the stability of the system (Morris et al., 2005; Ulanowicz et al., 2009; Grami et al., 2011; Niquil et al., 2011), consequences of nonlinearity in trophic interactions on system stability can be evaluated more directly in a dynamical model. When the trophic interaction between host phytoplankton and parasitic fungi is parameterized by a prey–predator type model with non-linear functional response (e.g., Holling type II functional response) (Miki et al., 2011) or more explicitly parameterized by a host-parasite type model with SIV formulation (susceptible host, infected host, and free-living vector; Gerla et al., 2013), food web dynamics are predicted to be less stable than a model with a simple Lotka-Volterra type prey–predator functional response (Miki et al., 2011). In addition, although the network analysis implied a stabilization of the system by fungi (Grami et al., 2011; Niquil et al., 2011), the dynamical model clearly demonstrated that the presence of the F-Z link (Figure S1, Miki et al., 2011) or the presence of a host–fungus interaction itself (**Figure 3**, Gerla et al., 2013) can destabilize the system, especially in eutrophic conditions. The dependency of fungal zoospore production rate on host physiology and activity (e.g., nutrient uptake rate) is also proposed as the destabilizing factor (Gerla et al., 2013).
3. *Contribution of the mycoloop to material fluxes:* the above consideration on system stability implies that it may be risky to assume that the roles of parasitic fungi in material fluxes estimated by steady state model equates to their role under non-steady state conditions. A non-steady state dynamic of the food web may be caused internally by nonlinear trophic interactions (as mentioned above) or externally forced by environmental fluctuations. A steady-state model is able to provide a snapshot estimate of fluxes in an ecosystem even under non-steady-state conditions if the instantaneous mass accumulation rate in each ecosystem component is not too large (quasi-steady

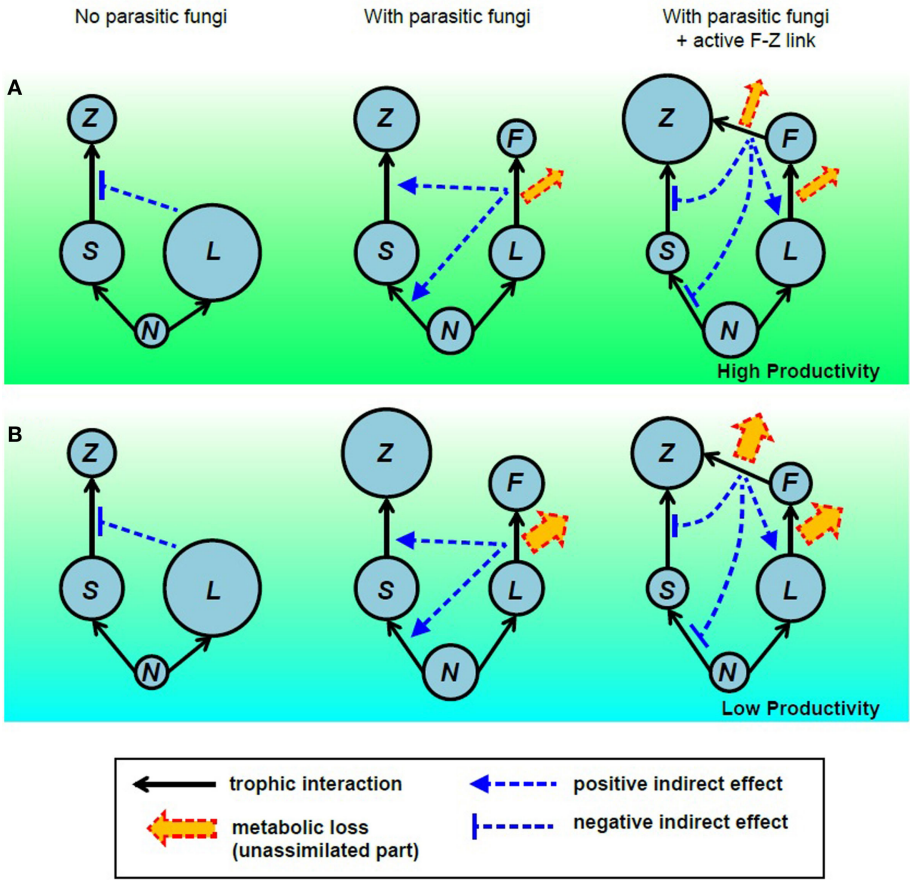

FIGURE 2 | Indirect effects of the F-Z link and their feedback on zooplankton. The net effects of the fungus–zooplankton link on zooplankton biomass production depends on three conditions: the growth efficiency of fungi on the host, the growth efficiency of zooplankton consuming fungi, and the nutrient supply in the system. ***N***, inorganic nutrient; ***L***, large phytoplankton; ***S***, small phytoplankton; ***F***, chytrid fungi; and ***Z***, zooplankton. **(A)** When these growth efficiencies are high, in other words, when the metabolic loss of fungi or metabolic loss of zooplankton is low, or the system productivity is high, the F-Z link increases zooplankton biomass production, compared to the system with fungal parasitism only. **(B)** When metabolic losses are high or the system productivity is low, the F-Z link decreases zooplankton biomass production compared to the system with fungal parasitism only. More quantitative results are shown in Miki et al. (2011).

state assumption). However, the steady state assumption tends to significantly overestimate (a factor of 2–10) the annual averaged contribution of the F-Z link under seasonal fluctuations (Miki et al., 2011; **Figure 3**). More specifically, the predicted relative contribution of fungi to zooplankton production from the dynamical model under a stable environment is 38.6% when the growth efficiency of fungi on host phytoplankton is assumed to be 75% and the total phosphorus is $100\,\mu gPL^{-1}$ (**Figure 3**). This prediction is comparable to the estimate of the contribution of fungal zoospores in the total diet of microzooplankton in ologimesotrophic Lake Pavin (38%) (Grami et al., 2011). However, an introduction of seasonality into the dynamical model lowers the contribution of fungi to 19.9%. Such an overestimate is a general feature in nonlinear systems. When the trophic flux (F) is proportional to the abundance of resources (R) and consumers (C): $F = aRC$ where a is the consumption coefficient, then the average flux $\left(\overline{F(t)}\right)$ is not equivalent to the product of the averages $\overline{R(t)}$ and $\overline{C(t)}$. Instead, we have $\overline{F(t)} = a\left[\overline{R(t)} \cdot \overline{C(t)} + \mathrm{Cov}\,(R(t), C(t))\right]$, implying that neglecting the impacts of asynchronous population dynamics of resources and consumers ($\mathrm{Cov}(R,C) < 0$) is the source of the overestimation with a steady-state assumption.

Combination of steady state models and dynamical models are a promising approach to greatly improve our understanding of the roles of parasitic fungi.

FUTURE PERSPECTIVES

MOLECULAR TOOLS

Recent advances in molecular methods enable us to investigate the species composition of microorganisms. Indeed, several methods, such as PCR-DGGE, clone libraries, FISH, and qPCR have been applied to describe the species composition and biomass of certain species of parasitic chytrids (Jobard et al., 2010; Lefèvre et al., 2010; Kagami et al., 2012; Marano et al., 2012, Maier et al., under revision). In addition, next generation sequencing

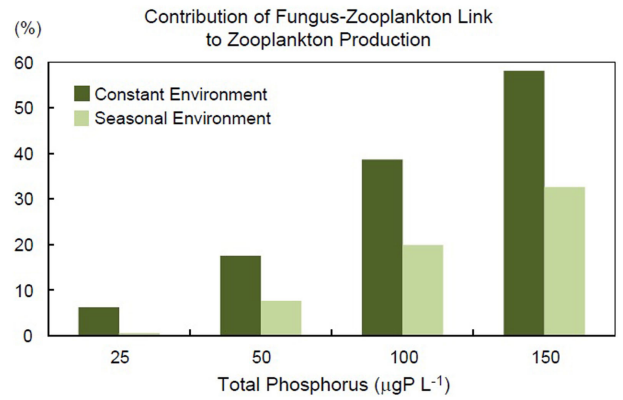

FIGURE 3 | Comparison of the contribution of the mycoloop pathway to zooplankton production under constant and seasonal environments. The relative percent contribution of the mycoloop pathway (large phytoplankton → parasitic fungi → zooplankton) to total zooplankton biomass production under constant and seasonal environmental conditions was calculated as the ratio of the F-Z link nutrient flux to zooplankton compared to the total nutrient flux to zooplankton (nutrient flux from the F-Z link plus nutrient flux from small phytoplankton) (see also **Figure 2**). The ratios of the contribution of the mycoloop under constant environmental conditions compared to that under seasonal environmental conditions was calculated for concentrations of total phosphorus (TP) = 25.0, 50.0, 100, 150 μgP L^{-1} to be 10.3, 2.30, 1.94, 1.78. TP in the model ecosystem was calculated by the average phosphorus supply I_0 divided by the turnover rate of the system (0.05/day). The daily fluctuation in the phosphorus supply $I(t)$ is given by $I(t) = I_0\left[1.0 + 0.5\sin(2\pi t/365)\right]$ for the seasonal environment; the maximum deviation from average is ±50%. Modified from Miki et al. (2011).

will be beneficial for the analysis of fungal community structures. However, since the DNA database of aquatic fungi is scarce, especially for the parasitic fungi (chytrids), it is difficult to determine species composition and function (e.g., parasitic or saprotrophic) by analyzing environmental DNA alone. In addition, choosing the right primer sets are essentially important when examining the species composition by molecular tools (Wurzbacher et al., 2010, Ishii et al., in review). Therefore, prior to applying advanced molecular tools, we first need to build a robust database, especially for the parasitic chytrids. Culturing, single cell PCR methods, and whole genome sequencing will aid in having a better understanding of the community structure and function of parasitic fungi wide-ranging ecosystems.

OTHER POSSIBLE MYCOLOOPS

In addition to parasitic chytrids, saprotrophic chytrids may also play important roles in aquatic food webs. For instance, pollen deposition into lakes may not be utilized directly by zooplankton, but can be decomposed/consumed by saprotrophic chytrids (Masclaux et al., 2011, 2013). Grazing of zoospores released from pollen may then function as another "mycoloop" (**Figure 4**).

Recently discovered fungi, the Cryptomycota, exhibit a similar life cycle to chytrids including a free-swimming stage, and are also known to infect phytoplankton (Jones et al., 2011). In marine environments, some Chytridiomycota or zoosporic fungal-like protists such as Labyrinthulomycota are also known to infect marine phytoplankton (Raghukumar, 2002; Gleason et al., 2011), and may play important roles in marine food web dynamics (Raghukumar, 2002). These results indicate the existence of

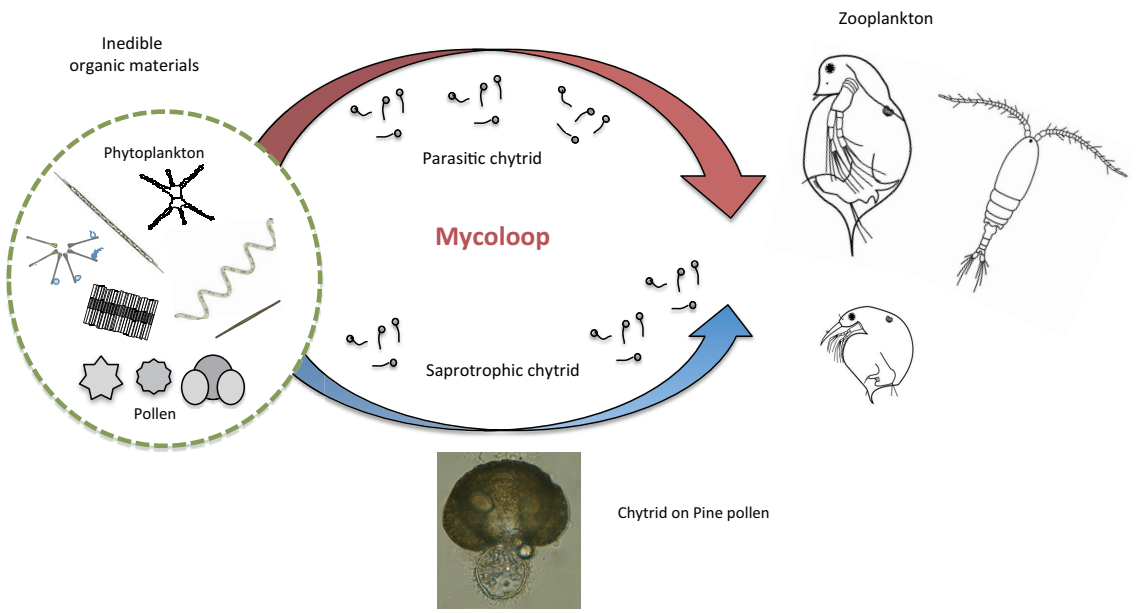

FIGURE 4 | Other possible mycoloops in freshwater and marine environments. Saprotrophic chytrids may play important roles in aquatic food webs, by decomposing inedible organic material such as pollens. Zoospores released from pollen may be consumed by zooplankton, functioning as another "mycoloop." In addition to chytrids, other zoosporic fungi or fungal-like protists, such as Cryptomycota and Labyrinthulomycota, can infect phytoplankton or consume large inedible organic material, which may be grazed by zooplankton in freshwater and marine environments.

other possible mycoloops in freshwater and marine ecosystems via the route of free-swimming zoospores of newly discovered Chytridiomycota, Cryptomycota, or Labyrinthulomycota.

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Parasitic chytrids sustain zooplankton growth during inedible algal bloom

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This study assesses the quantitative impact of parasitic chytrids on the planktonic food web of two contrasting freshwater lakes during different algal bloom situations. Carbon-based food web models were used to investigate the effects of chytrids during the spring diatom bloom in Lake Pavin (oligo-mesotrophic) and the autumn cyanobacteria bloom in Lake Aydat (eutrophic). Linear inverse modeling was employed to estimate undetermined flows in both lakes. The Monte Carlo Markov chain linear inverse modeling procedure provided estimates of the ranges of model-derived fluxes. Model results confirm recent theories on the impact of parasites on food web function through grazers and recyclers. During blooms of "inedible" algae (unexploited by planktonic herbivores), the epidemic growth of chytrids channeled 19–20% of the primary production in both lakes through the production of grazer exploitable zoospores. The parasitic throughput represented 50% and 57% of the zooplankton diet, respectively, in the oligo-mesotrophic and in the eutrophic lakes. Parasites also affected ecological network properties such as longer carbon path lengths and loop strength, and contributed to increase the stability of the aquatic food web, notably in the oligo-mesotrophic Lake Pavin.

Keywords: fungal parasites, bloom, stability, inverse modeling, ecological network analysis

INTRODUCTION

Parasites are known to be ubiquitous in their environments. Although they have been considered as important forcing factors for ecological processes (Hudson et al., 2006), they have only recently been included in food web studies. In aquatic ecosystems, freshwater parasites are especially common in the form of "zoosporic" fungi (i.e., chytrids). The life cycle of these parasites is characterized by dispersal forms, uniflagellated zoospores, and sporangia attached to the host cells. Microscopic observations provided evidence for the presence of both forms in freshwater ecosystems (Rasconi et al., 2009; Jobard et al., 2010). These parasites mostly affect primary producers (Canter, 1950; Sparrow, 1960), food web dynamics (Mccallum et al., 2004; Sime-Ngando, 2012) and ecological processes (Hudson et al., 2006). Many phytoplankton species are sensitive to chytrid parasites and the related ecological implications are important (Canter and Lund, 1948; Niquil et al., 2011; Sime-Ngando, 2012). Chytrid infections have been linked to mass mortalities of host organisms, to suppression or postponement of phytoplankton blooms, and have selective effects on host species composition and successions (Van Donk and Ringelberg, 1983; Bruning et al., 1992; Kagami et al., 2007; Rasconi et al., 2009). Unstable ecosystems seem to favor the opportunistic behavior of parasites (Rasconi et al., 2012), where their activity represents an important but as yet overlooked ecological driving force in food web dynamics. During blooms, parasites can optimize their virulence, as the host population is genetically more uniform (Brown et al., 2002) and bloom-forming species exhibit short generation time. Some fungal parasites seem to be most common in large and bloom forming algae that are fairly resistant to grazing by zooplankton (Sommer, 1987; Kagami et al., 2007; Rasconi et al., 2012), including diatoms and cyanobacteria. Recent studies have suggested a role for fungal parasites in destroying large filamentous phytoplankton, which are considered important for seasonal pelagic succession (Rasconi et al., 2012; Gerphagnon et al., 2013). This finding raises the hypothesis that parasites may play important roles during monospecific blooms of inedible algae because they can release dissolved substrates for microbial processes through host destruction, and provide energetic particles as zoospores for higher trophic levels (Kagami et al., 2007; Grami et al., 2011).

Diatoms traditionally form large spring blooms in temperate lakes, providing fuel for planktonic community development at

the start of the growing season. However, some of the diatom blooms are not grazed by filter-feeding zooplankton due to their large size, and this biomass is believed to be lost by sinking from the euphotic zone instead of being grazed. Recently there has also been an increasing awareness of food quality as a limiting factor for zooplankton growth (Brett and Muller Navarra, 1997; Sterner and Elser, 2009; Kagami et al., 2011). Laboratory studies suggest that a diatom monodiet lacks or is deficient in some essential component required for copepod egg development and may have a harmful effect on the success of egg hatching. Some authors have also highlighted the toxicity of these algae as food and presented evidence showing that the hatching success of wild copepods feeding on a diatom-dominated bloom is heavily compromised (Miralto et al., 1999). If diatoms have a deleterious effect, high diatom abundance could limit secondary production and affect fish production (Irigoien et al., 2002). However, diatoms have traditionally been regarded as providing the bulk of the food that sustains the planktonic seasonal succession and the food chain to top consumers (Sommer et al., 1986). Additionally, spring diatom proliferations are generally followed by a rapid increase in zooplankton. Diatoms are well known as preferential hosts for chytrid epidemics in the plankton (Ibelings et al., 2004; Kagami et al., 2007) and chytrid zoospores were experimentally demonstrated to be efficiently grazed and be able to sustain *Daphnia* growth in *Asterionella* cultures (Kagami et al., 2007). This implies that during fungal epidemics abundant zoospores may become a food source for some grazers. When fungi infect these large inedible phytoplankton species, they consume nutrients within these cells to produce zoospores, some of which are grazed by zooplankton with important consequences for the recycling of the organic matter in the pelagic food web (Kagami et al., 2007; Grami et al., 2011; Niquil et al., 2011).

Cyanobacteria, the most ancient phytoplankton on the planet, have been an important element for forming the earth's oxygen atmosphere. They frequently form blooms and dominate phytoplankton communities in warm, stratified and nutrient-enriched waters. In lakes, they form the basis of the food chain and function as nitrogen fixers. Proliferation events seem to have increased substantially during recent decades, likely as a result of eutrophication and temperature increase; they may have a large impact on water quality and biological communities. Many genera of cyanobacteria are known to produce a wide variety of toxins and bioactive compounds (Sivonen and Jones, 1999), which are a health risk to both animals and humans. From the perspective of aquatic food webs, cyanobacterial blooms can noticeably decrease the efficiency of the energy transfer from primary producers to primary consumers (Lurling and Roessink, 2006). However, filamentous cyanobacteria are known to be the target of different chytrid species (Canter, 1972). A recent hypothesis has been proposed stating that proliferation events may not always represent trophic bottlenecks, since eukaryotic parasites provide energetic particles as zoospores for higher trophic levels (Rasconi et al., 2012; Gerphagnon et al., 2013). Parasitism helps release dissolved substrates for microbial processes through host destruction.

Despite current evidence that bloom situations are considered deleterious for the ecosystem due to harmful and toxic species, blooms can constitute ecologically important events. They contribute to the natural processes of a lake and in some cases provide important benefits by boosting primary productivity and influence the energetics and population dynamics of consumer organisms. Considering the widespread occurrence of parasites during such situations, their consumption likely represents a far more important trophic link than previously recognized. Eukaryotic parasites as consumers drain energy throughout the food web and provide energetic particles for the grazers by predation on free living stages (Kagami et al., 2007; Grami et al., 2011; Miki et al., 2011). Parasitic activity was estimated to constitute between 36% and 44% of observed trophic links in a marine food web (Lafferty et al., 2006). In this paper we analyzed the role of parasitic chytrids and how they affect organic matter transfer in two different algal proliferation contexts: a spring diatom proliferation in an oligo-mesotrophic deep lake, and a late summer cyanobacterial bloom in a eutrophic shallow lake. The aim was to investigate how parasites drive energy and nutrients from their hosts to zooplankton in these two different situations to corroborate our recent findings that the activity of parasites and grazing on parasitic zoospores might sustain the growth of zooplankton through releasing nutrients bound in inedible algae and thus represent an important alternative carbon pathway in pelagic environments (Kagami et al., 2007; Grami et al., 2011; Miki et al., 2011).

Moreover, since we have demonstrated in a previous work (Grami et al., 2011) that parasites drive an increase in species richness, trophic level, connectance, and trophic chain length of the food web, we wanted also to establish the effects of parasites on the ecosystem properties linked to stability during monospecific algal proliferations. In this context of infections of primary producers, predation on parasites occurs at low trophic levels, which is considered a top down effect that reduce loop weight and increase the strength of links (Neutel et al., 2002). Weak to intermediate strength links are important as they can decrease complex oscillatory food-web dynamics and promote community persistence and stability (Mccann et al., 1998). Moreover, food-web stability is linked to species number and connectance, which is enhanced when species at high trophic levels feed on multiple prey or when species at intermediate trophic levels are fed on by multiple predator species (Gross et al., 2009). Parasites can alter topological properties of the network such as patterns of biodiversity, linkage between density and loop strengths, with implications for changing interactive networks and network stability (Lafferty et al., 2008). The significance of such observations is only beginning to be appreciated; integration of parasites has the potential to alter our understanding of food web structure and theory. The impact that parasites have on food web properties like stability and resilience has been previously overlooked and will need to be measured to ascertain its importance in the food web. From a theoretical perspective, predation on algal parasites helps to unite two emerging concepts in plankton ecology: the energetics of the overlooked parasite-grazer system flow during algal biomass proliferation and the impact of this link on the structural and functional properties of the ecosystem.

To investigate parasite-related flows, ecosystem properties and ecological theories, we applied mathematical tools such as linear inverse models for trophic network representation through

carbon flows. For the first time we evaluate and compare the impact of zoosporic parasites ("chytrids," class Chytridiomycetes, families Rhizophidiaceae and Chytridiaceae) on the functioning of a planktonic ecosystem using field data collected from the euphotic zone of two different lake ecosystems in the Massif Central region of France: the oligo-mesotrophic Lake Pavin and the eutrophic Lake Aydat. We compared carbon flows between the complete food web including parasitic chytrids during spring diatom bloom peak in Lake Pavin (April 2007 from 4th to 18th, PavDiat), with the model representative for the fall cyanobacteria bloom in Lake Aydat (from September 24th to October 10th 2007, AydCyan) and quantified the amount of primary production channeled through the food web. These models were built using the Linear Inverse Modeling procedure (LIM, Vezina and Platt, 1988) recently modified into the LIM-Monte Carlo Markov Chain (LIM-MCMC, Van Den Meersche et al., 2009). This method allows reconstruction of missing flow values and alleviates the problem of under-sampling, using the principle of conservation of mass (Vezina and Platt, 1988). The flows obtained from the models were used for calculations of Ecological Network Analysis indices that characterize the structure and functioning properties of the food web, and help reveal emergent properties (Ulanowicz, 1986, 1997; Ulanowicz et al., 2009).

LIM-MCMC and ecological network analysis were used to reveal overlooked trophic links in two contrasted freshwater ecosystems (Pavin and Aydat Lakes) where parasites of microalgae were integrated during bloom periods. Our objectives were to identify and compare (1) the carbon flows involved in the chytrid-parasitism pathway during monospecific algal proliferations in both lakes, (2) the emergent properties of different planktonic food webs during different algal blooms in which parasites are integrated, and (3) the structural and functional properties of two different ecosystems containing different parasite-host associations.

MATERIALS AND METHODS

STUDY SITE AND SAMPLING

Samples were collected in two freshwater lakes with different trophic status located in the French Massif Central. Lake Pavin (45° 29′ 41″ N, 002° 53′ 12″ E) is an oligo-mesotrophic, deep volcanic mountain lake ($Z_{max} = 92$ m), with a permanent anoxic monimolimnion from 60 m depth downwards. This site has a small surface area (44 ha), about equal to the drainage basin area (50 ha) and offers a unique environment with low human influences, and consistent annual seasonal dynamics in the water column (Lefèvre et al., 2007, 2008; Rasconi et al., 2012). Lake Aydat (45° 39′ 48″ N, 02° 59′ 04″ E) is a small eutrophic lake ($Z_{max} = 15$ m, surface area = 60 ha). Compared to the surface of the lake, the catchment area (3×10^4 ha) is very large and contains intensive agricultural lands.

Samples were collected fortnightly in a central location of each lake by simple capillarity as described by Sime-Ngando and Hartmann (1991). This method allowed collecting integrated samples (21 L) representative of the euphotic layers (0–20 m for Lake Pavin and 0–5 m for Lake Aydat). Samples from Lake Pavin were collected between 4 and 18 April 2007 (diatom bloom), while those considered for the Lake Aydat were collected between 24 September and 10 October 2007 (cyanobacteria bloom). Samples were pre-filtered on 150 μm pore size nylon filter (except for metazooplankton samples) for the elimination of metazoan zooplankton and taken to the laboratory for immediate analysis.

ABUNDANCE AND BIOMASS OF PLANKTONIC ORGANISMS

Sub-samples were processed for identification and quantification of picoplankton, heterotrophic nanoflagellates, phytoplankton, zooplankton and the two life stages of microphytoplankton fungal parasites (Chytridiales). Details on the material and methods are available in Grami et al. (2011).

Bacteria

Sample aliquots were fixed with glutaraldehyde before counting of heterotrophic and autotrophic picoplankton by a flow cytometer (BD system). Carbon conversion factors of 0.35 pg C μm^{-3} and 0.22 pg C μm^{-3} were used for conversion of biovolumes to carbon biomasses of heterotrophic bacteria (*bac*) (Bjornsen, 1986) and picophytoplankton (*ph1*, 0.2–2 μm) (Mullin et al., 1966; Sondergaard et al., 1991), respectively.

Heterotrophic nanoflagellates (hnf, 2–20 μm)

Sub-samples (15 ml) were fixed and handled according to Caron (1983) for quantification of heterotrophic nanoflagellates. Counts were performed using an inverted epifluorescent microscope (Leica DMIRB). Mean cell biovolumes were estimated for each sample by measuring the linear dimension of at least 50 cells and equating shapes to standard geometric forms. Carbon biomass was calculated using a conversion factor of 0.22 pg C μm^{-3} (Børsheim and Bratbak, 1987).

Nano- and microphytoplankton (ph2, 2–20 μm and ph3, 20–150 μm, respectively)

Sub-samples (200 ml) were fixed with alkaline Lugol solution (1% v/v) and cells were counted and identified using the Utermohl method (1931) under an inverted microscope (WILD—M40). Cell biovolumes were estimated by measuring the linear dimension of at least 100 cells and equating shapes to standard geometric forms. The resulting volumes were transformed into organic carbon values using the conversion equation of Menden-Deuer and Lessard (2000) (pgC $cell^{-1} = 0.288 \times Vol^{(0,811)}$ for diatoms and pgC $cell^{-1} = 0.216 \times Vol^{(0,939)}$ for the other autotrophic genera).

Ciliates (mic, 20–150 μm)

Sub-samples (200 ml) were fixed with alkaline Lugol solution (5% v/v) and ciliates were counted and identified using the same method as for microphytoplankton. For carbon biomasses of ciliates, biovolumes were converted into organic carbon using conversion factors of 0.19 pg C μm^{-3} (Putt and Stoecker, 1989).

Metazooplankton (mes)

The metazooplankton was collected by filtering raw samples from the euphotic layer (0–20 m) through a 50 μm pore-size mesh. Retained animals were preserved in 4% formalin-sucrose (Prepas, 1978). Identification and counting, after addition of few drops of

rose Bengal to improve detection, were conducted under a binocular microscope (Wild M3Z) using Dolfuss chambers (Dussart, 1967). The carbon biomass of each metazoan group was estimated by multiplying the individual carbon contents by the corresponding abundances. For Copepods the dry weight (DW, mg) was calculated as 22.5% of wet weight (Riemann et al., 1990; Gradinger et al., 1999) and C content (mg) was estimated as 40% of DW (Feller and Warwick, 1988). For Cladocera the length (L, mm) of each organism was used to determine its carbon content (C_{clad}) as: $\mu g\, C\, ind^{-1} = 5.24 \times L - 1.08$ (Kankaala and Johansson, 1986). For rotifers, wet weights were converted to dry weight according to Pace and Orcutt (1981) and Mccauley (1984). Dry weights were converted to carbon biomass using carbon: DW ratio of 0.48 (Andersen and Hessen, 1991).

Chytrid parasites

Sub-samples were handled for chytrid parasites counting based on a size fraction approach and the use of the fluorochrome calcofluor white (CFW) for diagnosing, staining and counting chitinaceous fungal parasites (i.e., sporangia of chytrids) of microphytoplankton (Rasconi et al., 2009). 20 L of the integrated samples were passed through a 25 μm pore size nylon filter. Large phytoplankton cells in the >25 μm size fraction were collected and fixed with formaldehyde (2% final conc.) before staining and analysis.

Nanoplanktonic cells in the <25 μm size-fraction were concentrated by ultrafiltration and 180 ml of the ultrafiltrate retentate was fixed with formaldehyde (2% final conc.), before staining and analysis. Aliquots (150 μl) of each fraction were stained by CFW (1% v/v) and drops (10 μl) of stained samples were mounted between glass slides and cover slips for observation and counting under an inverted epifluorescent microscope (more details are available in Rasconi et al., 2009). Identification of chytrids was based on phenotypic keys known from classical manuals, primarily those in Canter (1950); Canter and Lund (1951), and Sparrow (1960). The prevalence of infection was estimated as the percentage of infection in the host population according to Bush et al. (1997), i.e., $Pr\ (\%) = [(Ni/N) \times 100]$, where Ni is the number of infected host cells, and N is the total number of host cells. Carbon biomass of sporangia attached to the host cells (*spg*) was estimated using a conversion factor of 10.7 pg C $cell^{-1}$ (Kagami et al., 2007).

For zoospore (*zsp*) counting, sub-samples were processed using the CARD-FISH method of Not et al. (2002), recently modified by Jobard et al. (2010). The number of zoospores produced by sporangia was considered and carbon biomass of zoospores was estimated using a conversion factor of 10.7 pg C $cell^{-1}$ (Kagami et al., 2007).

MODEL CONSTRUCTION

Data from the field were used to construct pelagic food web models that quantitatively illustrate carbon pathways in Lake Pavin during diatom spring bloom and Lake Aydat during cyanobacteria autumn bloom, in the presence of chytrids (both sporangia and zoospore stages in the life cycle). Since the unknown flows far outnumbered the known flows, we adopted the LIM-MCMC method (Van Den Meersche et al., 2009), derived from the LIM of Vezina and Platt (1988) to reconstruct trophic flows through the pelagic food web. The approach is based on four steps described with more details in Grami et al. (2011).

Compartments and a priori model

The first step consists in constructing a conceptual model including all possible flows between compartments and between compartments and the outside. We represented the pelagic food web and carbon pathways in the mycoloop, we thus did not include compartments for fishes and macrophytes. Living compartments included three phytoplankton compartments, three grazer compartments, one compartment for heterotrophic bacteria and two compartments for fungal parasites of microphytoplankton. We divided the phytoplankton into picophytoplankton (*ph1*: 0.2–2 μm); nanophytoplankton (*ph2*: 2–20 μm; principally Cryptophyta as *Rhodomonas* sp. in both lakes and Chlorophyta as *Ankistrodesmus sp.* and *Ankyra sp*. in Lake Pavin) and microphytoplankton (*ph3*: 20–150 μm; essentially large and filamentous Bacillariophyceae, as *Synedra* sp., *Melosira* sp., and *Asterionella* sp. in Lake Pavin and filamentous cyanobacteria as *Anabaena* sp. and *Oscillatoria* sp. in Lake Aydat). Grazer compartments were the heterotrophic nanoflagellates (*hnf*: 2–20 μm), microzooplankton (*mic*; 20–150 μm: Ciliates and small Rotifera) and mesozooplankton (*mes*; >150 mm; Cladocera, Copepoda and some large Rotifera). Phytoplankton fungal parasites compartments included sporangia attached to the host cells (*spg*) and free zoospores (*zsp*). Non-living compartments were dissolved organic carbon (*doc*) and detritus (*det*).

The food web contained 53 carbon flows for the model of Lake Pavin and 54 for the model of Lake Aydat (a flow was added allowing the microzooplankton to consume detrital material). The sole carbon inputs were gross primary production by each phytoplankton size fraction. Carbon output from the network was driven by respiration of all living compartment and carbon loss by sinking from *ph2*, *ph3*, *spg*, *mic*, *mes,* and *det* compartments. Mesozooplankton contribution to the carbon output flow considers their consumption by higher trophic level and their production of sinking fecal pellets. All living compartments except fungal parasites contribute to the DOC production that was taken up by bacteria. In addition to *ph2*, *ph3*, *mic,* and *mes* contribution to detritus production, we considered the existence of a carbon flow from bacteria and heterotrophic nanoflagellates to detritus.

Attached bacteria were identified on TEP—Transparent Exopolymer Particles, associated with vertical flows of carbon in Lake Pavin during spring (Carrias et al., 1998), and then a flow of bacteria to detritus was considered. The bacteria to detritus flow was calculated using data on bacteria attached to TEP sedimenting in Lake Pavin during spring (Lemarchand et al., 2006). These attached bacteria are known to constitute preferential prey for heterotrophic nanoflagellates (Arnous et al., 2010). Detritus production of sporangia was due to chitinaceous wall dissolution or break-up during zoospore discharge (Sparrow, 1960). Moreover, zoospores were considered as contributing to detritus production by the loss of their flagellum when they found a host to fix on. The carbon flow from microphytoplankton to sporangia represented carbon pumped from diatom cells to sporangia

and the carbon flow from sporangia to zoospores was considered as the zoospores produced by sporangia. Grazing relationships were defined by considering size and preferential ingestion of each identified grazer. Heterotrophic flagellates grazed on *bac* and *ph1*, microzooplankton grazed on *bac*, *ph1*, *ph2*, *ph3*, *hnf, zsp,* and *det* (this latter only in Lake Aydat) and mesozooplankton grazed on *bac*, *ph1*, *ph2*, *ph3*, *hnf*, *mic*, *zsp,* and *det*.

Equalities

The second step was setting equations (equalities) to constrain the mass balance of the system and to impose measured flows. The mass balance equations for all compartments are given in the first 11 lines of the **Table 1**. Some of the estimated flows were measured during previous studies that focused on spring blooms in Lake Pavin and are introduced as additional equations (lines 12–15 of **Table 1**); these include values for total gross and net primary production (Devaux, 1980; Bettarel et al., 2003), bacterial production (Bettarel et al., 2003) and viral lysis of bacteria (Bettarel et al., 2003) considered as the value of the flux from bacteria to DOC. Some other equalities were introduced for the Aydat model and values of total gross primary production (Aleya et al., 1988), bacterivory by heterotrophic nanoflagellates and microzooplankton (Bettarel et al., 2004) were considered (lines 16–18 of **Table 1**). Primary production values used for Pavin and Aydat were measured from 14C uptake according to Steemann-Nielsen (1952). Bacterial production was determined by measuring the uptake of tritiated thymidine into bacterial DNA (Petit et al., 1999), after incubating the samples for 45 min. Viral lysis of bacteria was considered as the value of the flux from bacteria to DOC. The fraction of bacterial mortality from viral lysis was related to the calculated frequency of visibly infected cells, calculations are detailed in Bettarel et al. (2003). Details on the method are available in Grami et al. (2011) and cited references there in. Values of protozoan bacterivory were calculated using tracer particles and epifluorescence microscopy (EM) following (Pace and Bailiff, 1987) method modified by Carrias et al. (1996), more details are available in Bettarel et al. (2004).

Table 1 | Mass balance (1–11) and linear equations used for inverse analysis.

Equation number	Process concerned	Equations
COMMON MASS BALANCE EQUATIONS BETWEEN PAVIN AND AYDAT MODELS		
1	Mass balance for microphytoplankton	(gpp-ph3) − (ph3-res + ph3-doc + ph3-mic + ph3-mes + ph3-spg+ ph3-det + ph3-los) = 0
2	Mass balance for nanophytoplankton	(gpp-ph2) − (ph2-res + ph2-doc + ph2-mic + ph2-mes + ph2-los + ph2-det) = 0
3	Mass balance for picophytoplankton	(gpp-ph1) − (ph1-res + ph1-doc + ph1-hnf + ph1-mic + ph1-mes+) = 0
4	Mass balance for heterotrophic nanoflagellates	(ph1-hnf + bac-hnf) − (hnf-res + hnf-doc + hnf-mic+ hnf-mes + hnf-det) = 0
5	Mass balance for bacteria	(doc-bac) − (bac-res + bac-doc + bac-hnf + bac-mic + bac-mes + bac-det) = 0
6	Mass balance for microzooplankton	(ph1-mic+ ph2-mic + ph3-mic + bac-mic + hnf-mic + det-mic+ zsp-mic) − (mic-res + mic-doc + mic-mes + mic-det+ mic-los) = 0
7	Mass balance for mesozooplankton	(ph1-mes+ ph2-mes + ph3-mes + bac-mes + hnf-mes + mic-mes +det-mes + zsp-mes) − (mes-res + mes-doc + mes-det + mes-los) = 0
8	Mass balance for sporangia	(ph3-spg) − (spg-res + spg-zsp + spg-det+ spg-los) = 0
9	Mass balance for zoospores	(spg-zsp) − (zsp-res + zsp-mic + zsp-mes + zsp-det) = 0
10	Mass balance for detritus	(ph2-det + ph3-det + hnf-det + mic-det + mes-det + bac-det + spg-det + zsp-det) − (det-doc + det-mic**+ det-mes + det-los) = 0
11	Mass balance for dissolved organic carbon	(ph1-doc + ph2-doc + ph3-doc + hnf-doc + mic-doc + mes-doc + det-doc) − (doc-bac) = 0
PAVIN LINEAR EQUALITIES		
12	Total gross primary production estimate	gpp-ph1 + gpp-ph2 + gpp-ph3 = 676.25*
13	Total net primary production estimate	(gpp-ph1 + gpp-ph2 + gpp-ph3) - (ph1-res + ph2-res + ph3-res) = 459.85*
14	Net bacterial production	doc-bac − bac-res = 105.60*
15	Viral lysis of bacteria	bac-doc = 9.90*
AYDAT LINEAR EQUALITIES		
16	Total gross primary production estimate	gpp-ph1 + gpp-ph2 + gpp-ph3 = 2520.00*
17	Bacterivory by microzooplankton	Bac-hnf = 109.40*
18	Bacterivory by heterotrophic nanoflagellates	Bac-mic = 63.02*

Gpp-compartment A: gross primary production of compartment A.

Compartment A-Compartment B (e.g., Bac-hnf) represent the carbon flowing from compartment A to compartment B.

Compartment A-res: respiration of compartment A.

Compartment A-los: carbon loss by sedimentation of compartment A.

Bac, bacteria; Ph1, picophytoplankton; Ph2, nanophytoplankton; Ph3, microphytoplankton; Hnf, heterotrophic nanoflagellate; Mic, microzooplankton; Mes, mesozooplankton; Spg, sporangia; Zsp, zoospores; Det, detritus; Doc, dissolved organic carbon.

**values are in mgC m^{-2} d^{-1}.*

***det-mic was only considered for Aydat model.*

Constraints

The third step consisted of imposing ecological limits (maximum and/or minimum) for each unknown flow, which means a linear system of inequalities: 76 inequalities where provided for Lake Pavin and 78 inequalities for Lake Aydat. The latter is presented, explained and referenced in **Table 2**. Details about these inequalities are given in Grami et al. (2011).

Solutions

The last step of the inverse analysis was the calculation of flows. A range of possible values was given by the method of the Monte Carlo Markov Chain joined to the mirror technique (Van Den Meersche et al., 2009). A jump value of 10 mgC $m^{-2}d^{-1}$ and 100,000 iterations were used to cover all possible solutions. More details on this method are available in Van Den Meersche et al. (2009) and Niquil et al. (2012).

ECOLOGICAL NETWORK ANALYSIS

The resulting flows issued from inverse analysis, together with estimated biomasses, were used for calculating Ecological Network Analysis indices in order to describe the emergent properties of the ecosystem.

Total system throughput (TST) represents a measure of the total system activity and is the sum of all the flows through all compartments (Kay et al., 1989).

Average path length (APL) is the average number of compartments crossed by a unit of carbon from its entry to the system to its leaving. It represents a measure of the system retention (Kay et al., 1989).

System ascendency (A) is a measure of the system size and organization. It is the product of the TST and the average mutual information (AMI: degree of specialization of flows in the network) (Ulanowicz, 1986). This value is more informative about the organization of the system when it is expressed in relation to development capacity and considered as its maximum value (A/DC). It defines the ecosystem degree of development. High Relative ascendency indicates more specialized and less redundant pathways. The structure of energy flows can be related to the concept of structural asymmetry and ecosystem stability (Rooney et al., 2006), because the pattern of asymmetric channel flow enhances the equilibrium stability of an ecosystem. The difference between the development capacity and the ascendency is called redundancy (R), which is a quantification of the multiplicity of parallel flows. The relative redundancy (R/DC, %) is a measure of the ecosystem degree of information loss due to parallel pathways. The system would be redundant when the ascendency is low.

Development capacity (DC) is calculated as the product of TST and the upper limit of AMI, corresponding to the maximum potential ascendancy and to a food web with maximum specialization. The development capacity is the sum of ascendency, redundancy and information loss related to external exchanges.

In this study, as suggested by Ulanowicz (1986), growth and development were characterized by indices calculated over only internal exchanges. We consider the internal capacity of ecosystem development (DCi), i.e., the sum of internal ascendency (Ai) and internal redundancy (Ri). The internal relative ascendency Ai/DCi could point to a strong dependency of an ecosystem on external inputs (Baird and Heymans, 1996) in case it decreases in relation to the A/DC ratio. However, as pointed out by Baird et al. (1991), Ai/DCi ratio could be an aspect of a highly organized ecosystem. The internal relative redundancy (Ri/DCi) is considered as a measure of ecosystem stability by many authors (Rutledge et al., 1976; Baird et al., 1998, 2004).

Finn Cycling Index (FCI) is the ratio of carbon flowing in loops (the carbon comes back to the compartment it left) to the sum of all carbon flows. I.e., it is the fraction of all flows involved in recycling (Finn, 1976) and can also be considered a measure of the retentiveness of a system.

Connectance measures the trophic connections within a food web. The overall connectance includes the effects of all transfers (exogenous and endogenous exchanges). The intercompartmental connectance characterizes only the endogenous exchanges. The food-web connectance pertains only to transfers among the living compartments (Ulanowicz, 2003).

Comprehensive Cycling Index (CCI) was proposed as a new index that gives the real importance of cycling after added corrections to the FCI. This new index considers four types of pathways that energy and matter can follow to join one compartment to another (Allesina and Ulanowicz, 2004).

Trophic analysis maps the complex network of trophic transfers as a linear food chain (called Lindeman spine, Ulanowicz and Kemp, 1979) based on the trophic concept of Lindeman (Lindeman, 1942). The Lindeman spine allows calculation of the trophic efficiency for each level, also called transfer efficiency (Ulanowicz and Wulff, 1991). The global trophic efficiency (Geff) is computed as the logarithmic mean of all the trophic level efficiencies. Two more indices were derived from the Lindeman spine: the grazing chain efficiency and the percentage of detritivory. The mean values of the 100,000 set of flows resulting from the LIM-MCMC analysis were used to build the Lindeman spine using the WAND software.

Detritivory/Herbivory ratio (D/H) is the sum of consumption of non-living material (detritus or DOC) divided by the sum of flows of consumption of autotrophic organisms. Parasitism was included in herbivory flow.

For these calculations, we used an algorithm written for Matlab© by Carole Lebreton and Markus Schartau (pers. comm.) to calculate the first set of indices: TST, APL, A, DC, R, Ri and connectance. 100,000 iterations of these ecological indices were computed allowing statistical analysis of the difference between those calculated for Lake Pavin and Lake Aydat. Recycling, connectance and trophic efficiencies indices were calculated using the ecological network analysis package WAND© by Allesina and Bondavalli (2004) available at http://www.dsa.unipr.it/netanalysis/?Software. Only one value index was obtained for these indices, based on the average flow value for each flow of the food-web, preventing us from statistically testing the difference between the two blooms.

DATA ANALYSIS

The statistical comparison between the two situations described, concerning the set of indices calculated under Matlab (TST, APL, A, DC, R, Ri and connectance) was tested with two tailed Student (t) tests using XLSTAT©.

Table 2 | Constraints used on different planktonic food web processes.

Process		Bound	Description	Equations	References
Gross primary production	ph3	Upper and lower	GPP of ph3 is comprised between 60% and 85% of total GPP	60% GPP < GPP-ph3 < 85% GPP	This study
	ph2	Upper and lower	GPP of ph2 is comprised between 2% and 10% of total GPP	2% GPP < GPP-ph2 < 10% GPP	
	ph1	Upper and lower	GPP of ph1 is comprised between 5% and 20% of total GPP	5% GPP < GPP-ph1 < 20% GPP	
Respiration	ph1 ph2	Upper and lower	ph2 and ph1 respiration is comprised between 5% and 30% of their GPP	50% GPP < R <30% GPP	Vezina and Platt, 1988
	ph3	Upper and lower	ph3 respiration is comprised between 5% and 40% of their GPP	50% GPP < R < 40% GPP	Vezina and Platt, 1988
	bac	Lower	Bacteria respiration is at least 20% of their total uptake of doc	20% U_{DOC} < R	Vezina and Savenkoff, 1999
	hnf, mic mes	Lower	Zooplankton respiration is at least 20% of their total ingestion and doesn't exceed their maximum specific respiration	20% ΣIng < R	Vezina and Savenkoff, 1999
	Chytrids	Upper	Sporangia and zoospores respiration doesn't exceed 20% of their carbon input	R < 20% C input	This study
Doc production	ph1, ph2 ph3	Upper and lower	Phytoplankton doc exudation is comprised between 10% and 55% of the net primary production (NPP)	10% NPP < E < 55% NPP	Breed et al., 2004
	hnf, mic mes	Upper and lower	Zooplankton exudation of doc is at least 10% of their total ingestion and doesn't exceed their respiration	10% ΣIng < E < R	Vezina and Pace, 1994
Growth efficiency	hnf, mic mes	Upper and lower	The growth efficiency is no more than 50% of the total ingestion (Ing) and is at least 25% of it	25% ΣIng < Ing − (R + E + Det) < 50% ΣIng	Vezina et al., 2000
	bac	Upper and lower	Growth efficiency of bacteria is comprised between 25 % and 50%	0.5ΣIng < R < 0.75ΣIng	Vezina and Pahlow, 2003
Assimilation efficiency	hnf, mic mes	Upper and lower	Assimilation efficiency of zooplanctonic compartments is comprised between 50 % and 90% of their ingestion	50% ΣIng < Ing -Det < 90% ΣIng	Vezina et al., 2000
Grazing of ph3 by mes		Upper and lower	ph3 grazing by mes is comprised between 3% and 7% of its net primary production	3% NPP-ph3< $Ing_{ph3-mes}$ < 7% NPP-ph3	Quiblier-Loberas et al., 1996
Predation on mic by mes		Upper	80% of total ingestion of mesozooplankton	$Ing_{mic-mes}$ < 0.8 ΣIng_{mes}	Vezina et al., 2000
Preferential ingestion of mes	bac	Upper and lower	Bacteria consumption by mes is comprised between 10 and 15% of mes total ingestion	10%ΣIng mes < Ing bac-mes < 15% ΣIng mes	This study

(Continued)

Table 2 | Continued

Process		Bound	Description	Equations	References
	ph2	Upper and lower	ph2 grazing by mes is comprised between 10 and 15% of mes total ingestion	10% Σlng mes < lng ph2-mes < 15% Σlng mes	
	hnf zsp	Upper and lower	The sum of hnf and zsp consumption by mes is comprised between 15 and 25% of mes total ingestion	15% Σlng mes< lng hnf+zsp-mes < 25% Σlng mes	
	mic	Upper and lower	Predation of mes on mic is comprised between 40 and 60% of mes total ingestion	40% Σlng mes < lng mic-mes < 60% Σlng mes	
Preferential ingestion of mic	bac ph1	Upper and lower	The sum of bac and ph1 consumption by mic is comprised between 10 and 15% of mic total ingestion	10% Σlng mic < lng bac+ph1-mic < 15% Σlng mic	This study
	ph2	Upper and lower	ph2 grazing by mic is comprised between 20 and 30% of mic total ingestion	20% Σlng mic < lng ph2-mic < 30% Σlng mic	
	hnf zsp	Upper and lower	The sum of hnf and zsp consumption by mic is comprised between 40 and 60% of mic total ingestion	40% Σlng mic < lng hnf + zsp-mic < 60% Σlng mic	
Preferential ingestion of hnf	bac	Lower	bac consumption by hnf is at least 60% of hnf total ingestion	60% Σlng hnf < lng bac-hnf	This study modified from Bettarel et al., 2003
	ph1	Lower	ph1 consumption by hnf is at least 20% of hnf total ingestion	20% Σlng hnf < lng ph1-hnf	
Detritus production	hnf	Upper	hnf contribution to det carbon input doesn't exceed 20% of its total ingestion	hnf-det < 20% Σlng_{hnf}	Carrias et al., 1998
	mes	Upper	mes contribution to det carbon input doesn't exceed 20% of its total ingestion	mes-det < 20% Σlng_{mes}	
	bac	Upper and lower	Between 1.2% et 5.6% of bacterial production (BP) will contribute to the det carbon input (Attached bacteria)	1.2% BP < Bac -Det < 5.6% BP	Lemarchand et al., 2006
	ph3	Upper and lower	Microphytoplankton det production is comprised between 16% and 95% of total det production	16% Σ Det < ph3-det < 95% Σ Det	Arnous et al., 2010
	Chytrids	Upper	Det production by sporangia exceed 5% of its carbon input	Det_{spg} < 5% GPP3-spg	Niquil et al., 2011
Detritus consumption by mes		Upper	Mes consumption of detrital is no more than 40% of detritus production	$lng_{det-mes}$ < 40% Σ Det	This study
Detritus dissolution		Upper	The upper bound of det dissolution is 10% of net particular production	10% NPP < det-doc	Pace et al., 1984
Zoospores ingestion		Lower	Zoospora ingestion by mic is at least twice its ingestion by mes	$lng_{zsp-mic}$ > 2 $lng_{zsp-mes}$	This study
Carbon transfer from microphytoplankton to host-attached sporangia		Lower	The lower bound of carbon transfered to sporangia after infections of ph3 cells is 8% of net particular production	gpp-ph3 TO spg > 8% NPP-ph3	This study modified from Kagami et al., 2006

(Continued)

Table 2 | Continued

Process		Bound	Description	Equations	References
Carbon transfert from sporangia to zoospores		Lower	The lower bound of carbon transfered from sporangia to zoospores is at least the carbon biomass of zoospores compartment	spg TO zsp > Biom zsp	This study
Sinking/loss	ph3	Lower	ph3 sinking is at least 28% of total carbon sinking	ph3-los > 28% Σlos	Kagami et al., 2002
			Sedimentation of ph3 exceed 0.2 mgC m^{-2} d^{-1}	ph3-los > 0.2	Carrias et al., 1998
	mes	Upper and lower	Sedimentation of mes range between 45% and 65% of total sedimentation	45% Σloss < mes-loss < 65% Σloss	Vezina et al., 2000

RESULTS

FLOW ANALYSIS

The values for overall flows for both models are given in **Table 3**. Carbon input into the lakes was only due to primary production. No allochthonous detritus input was considered due to the small catchment area (50 ha) of Lake Pavin and the low rainfall during the study period in Lake Aydat. Due to blooms of large algae in Lake Pavin (*Synedra* sp., *Melosira* sp., *Cyclotella* sp., and *Asterionella* sp.) and filamentous cyanobacteria in Lake Aydat (*Anabaena* sp. and *Oscillatoria* sp.) the major contribution to the gross primary production was provided by microphytoplankton (75% and 89% in Lake Pavin and Aydat, respectively). Calculated carbon throughput (T) of microphytoplankton was higher in Lake Aydat (2243 $mgCm^{-2}d^{-1}$). Consequently, all throughputs (and primarily those directly affected by primary production) were more important in Lake Aydat (**Figure 1**). According to the trophic status of the lakes, the contribution of picophytoplankton production to total gross primary production was higher in the oligo-mesotrophic Lake Pavin (14.7%) compared to the eutrophic Lake Aydat (1%).

The flows calculated in mgC $m^{-2}d^{-1}$ by the LIM—MCMC method directly involved in fungal compartments are detailed in **Figure 2**. In Lake Pavin, 9.6% of total system throughput was channeled to the sporangia parasitic compartment, which corresponded to 36.8% of microphytoplankton production. The sporangia compartment channeled 75% of its carbon input to the zoospore compartment. In Lake Aydat, the calculated percentage of the total system throughput values channeled to the sporangia compartment was slightly higher than in Lake Pavin and represented 11.7% (33% of *ph3* gross primary production). However, the sporangia compartment channeled less carbon to the zoospores compartment (67.6%). This was linked to a major sporangia loss flow in Lake Aydat (22.4% of total sporangia throughput) compared to Lake Pavin (only 5.5%).

Carbon flows involved in parasitism were of major importance for grazer compartments (i.e., micro- and mesozooplankton). Major zoospore throughput was channeled to the microzooplankton compartment (62.4% and 83.7%, respectively for Pavin and Aydat) and only 14.2% and 5% of zoospore throughput was ingested by the mesozooplankton compartment (**Figure 2**). Zoospores constituted the major nutritional resource for microzooplankton in Lake Pavin (50.5%) and in Lake Aydat (57.7%) (**Figure 3**). However, since microzooplankton represented the main ingested prey by mesozooplankton in both lakes (about 40–41%), carbon that originated from microphytoplankton parasitism was indirectly channeled to mesozooplankton through its ingestion of major microzooplankton throughput (**Figure 2**).

Moreover, carbon indirectly channeled from microphytoplankton to grazers through ingestion of zoospores produced by parasites' sporangia, represented 19–21% of microphytoplankton gross primary production in both lakes (**Figure 2**). This corresponded to 57–60% of sporangia throughput transferred to grazer compartments.

Sedimentation flows were lower in Lake Pavin compared to Lake Aydat, and represented 25% and 61.7% of the total system throughput, respectively. The largest contribution to this loss flow in Lake Aydat originated from microphytoplankton (57.7%, **Table 4**) while it originated from mesozooplankton (46.5% of total system loss, **Table 4**) in Lake Pavin. Detritus production originated mainly from microphytoplankton in the Aydat ecosystem (60% of total detritus throughput, **Table 4**) while in Lake Pavin zooplankton was the main contributor (48.2%, **Table 4**).

ECOLOGICAL NETWORK ANALYSIS

The LIM-MCMC derived flow results of each model were used to calculate the ecological network indices. **Table 5** provides comparisons of the most relevant ecological network analysis indices for both lakes. Significant differences ($P < 0.05$) in network properties of the two considered webs were observed for TST, DC, A, A/DC, DCi, Ai and Ri. No relevant significant difference was detected between both lakes for APL, AMI, R/DC, Ai/DCi and Ri/DCi.

The total system throughput (TST, **Table 5**) was much lower in Lake Pavin compared to Lake Aydat due to higher primary production and carbon input during the cyanobacteria bloom. The calculated averages were 2625 and 8889 $mgCm^{-2}d^{-1}$, respectively, for Pavin and Aydat.

The average mutual information (AMI) values gives information about flow specialization. Values of AMI were almost the same for Aydat and Pavin (1.94 for Aydat and 1.96 for Pavin).

Table 3 | Flow description, name and corresponding value (mg C m^{-2} d^{-1}) of steady state models of the pelagic food web of Lake Pavin and Lake Aydat during each lake bloom period.

Flow description	Flow name	Pavin spring bloom	Aydat Autumn bloom
		Inferred value (mg C m^{-2} d^{-1})	
		Model I	Model II
Microphytoplankton gross primary production	gpp-ph3	*509.35*	*2243.38*
Nanophytoplankton gross primary production	gpp-ph2	*66.62*	*253.91*
Picophytoplankton gross primary production	gpp-ph1	*100.28*	*22.70*
Microphytoplankton respiration	ph3-res	192.25	112.62
Microphytoplankton doc excretion	ph3-doc	39.10	193.94
Microphytoplankton grazing by mic	ph3-mic	13.25	0.26
Microphytoplankton grazing by mes	ph3-mes	11.89	64.05
Parasitism of ph3 by sporangia	ph3-spg	*187.44*	*746.00*
Microphytoplankton det production	ph3-det	15.50	228.93
Microphytoplankton sinking	ph3-los	*49.92*	897.58
Nanophytoplankton respiration	ph2-res	4.29	15.42
Nanophytoplankton doc excretion	ph2-doc	6.63	24.20
Nanophytoplankton grazing by mic	ph2-mic	35.74	184.98
Nanophytoplankton grazing by mes	ph2-mes	18.53	26.69
Nanophytoplankton sinking	ph2-los	*0.74*	2.48
Nanophytoplankton det production	ph2-det	0.70	0.14
Picophytoplankton respiration	ph1-res	19.86	2.58
Picophytoplankton doc excretion	ph1-doc	23.92	3.16
Picophytoplankton grazing by hnf	ph1-hnf	34.57	16.53
Picophytoplankton grazing by mic	ph1-mic	16.60	0.18
Picophytoplankton grazing by mes	ph1-mes	5.34	0.26
Bacteria respiration	bac-res	98.85	327.02
Bacterivory by hnf	bac-hnf	66.39	*63.02*
Bacteria uptake by mes	bac-mes	20.74	26.63
Bacteria uptake by mic	bac-mic	6.47	*109.40*
Bacterial doc release due to viruses lysis	bac-doc	9.90	0.71
Attached bacteria to det	bac-det	2.09	44.00
Heterotrophic nanoplankton respiration	hnf-res	38.12	27.37
Heterotrophic nanoplankton doc excretion	hnf-doc	21.56	14.55
Heterotrophic nanoplankton uptake by mic	hnf-mic	13.97	14.74
Heterotrophic nanoplankton uptake by mes	hnf-mes	14.65	14.78
Heterotrophic nanoplankton det production	hnf-det	12.66	8.11
Microzooplankton respiration	mic-res	47.82	255.55
Microzooplankton doc excretion	mic-doc	29.85	145.81
Microzooplankton uptake by mes	mic-mes	73.64	105.98
Microzooplankton egestion	mic-det	19.85	73.33
Microzooplankton sinking	mic-los	2.54	151.10
Mesozooplankton respiration	mes-res	48.57	93.11
Mesozooplankton doc excretion	mes-doc	30.49	50.08
Mesozooplankton egestion	mes-det	20.29	26.57
Mesozooplankton grazing by larger organisms	mes-los	78.64	94.42
Sporangia respiration	spg-res	32.93	74.86
Sporangia emission of zoospores	spg-zsp	*140.45*	*504.12*
Sporangia detrital production	spg-det	3.75	0.15
Sporangia sinking	spg-los	10.31	166.86
Zoospores respiration	zsp-res	24.46	56.94
Zoospores ingestion by mic	zsp-mic	87.68	421.87
Zoospores ingestion by mes	zsp-mes	19.92	25.15
Zoospores detrital production	zsp-det	8.40	0.15
Dissolved organic carbon uptake by bacteria	doc-bac	204.45	570.78
Detritus dissolution	det-doc	43.00	138.34
Detritus consumption by mes	det-mes	13.29	0.65
Detritus consumption by mic	det-mic	–	0.35
Detritus sinking	det-los	26.95	242.06

Italic values indicate flows that were estimated or derived from processes determined in situ. The rest are values constrained by one or two inequations and estimated by the LIM-MCMC method.

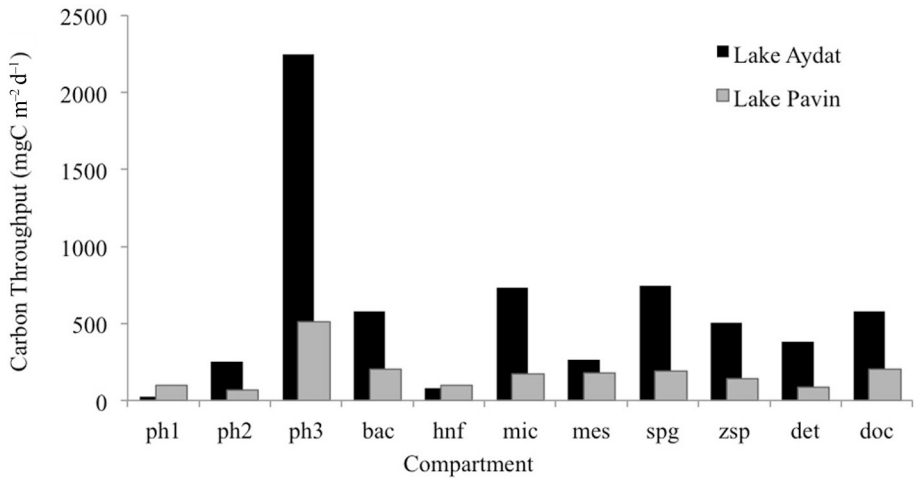

FIGURE 1 | Throughput of each compartment for both models (mgC m^{-2} d^{-1}): Pavin Spring bloom model (white chart) and Aydat Autumn bloom model (black chart). Bac, bacteria; Ph1, picophytoplankton; Ph2, nanophytoplankton; Ph3, microphytoplankton; Hnf, heterotrophic nanoflagellate; Mic, microzooplankton; Mes, mesozooplankton; Spg, sporangia; Zsp, zoospores; Det, detritus; Doc, dissolved organic carbon.

The average pathway length (APL), a measure of the system retention capacity, was not significantly higher in Lake Pavin (**Table 5**). The calculated mean number of compartments through which each inflow passes was 2.9 in Lake Pavin and 2.5 in Lake Aydat.

The topological indices, which characterize the ecosystem structure, were affected by the different carbon input in different trophic situations of the two lakes and were lower in Lake Pavin compared to Lake Aydat except for redundancy. The development capacity was higher for Lake Aydat (**Table 5**) following the pattern of higher TST due to cyanobacteria bloom. Relative ascendency (A/DC%) also was affected by higher TST in the eutrophic lake, being around 66% in Aydat and 61% in Pavin. System inefficiency due to internal parallel pathways, termed relative redundancy (R/DC), was higher, but not significantly, in Lake Pavin during the spring bloom (**Table 5**). Internal indices calculated over only internal exchanges showed higher internal ascendency (Ai) and internal redundancy (Ri) for the Lake Aydat, due to the presence of populations more adapted to rapid changes and the effect of parasites helping to restablish broken pathways more easily. But, relative internal or functional redundancy (Ri/DCi) was slightly more important for Lake Pavin (56%) due to the similar effect of parasites over the internal exchanges.

The overall connectance, number of trophic connections, within each food web including exogenous transfers, was higher for Lake Pavin (2.2 vs. 1.9) due to the effect of parasites in reducing the losses of organic matter and enhancing the transfers through the mycoloop. Even when only based on endogenous exchanges, the amount of trophic connections given by the intercompartmental connectance index was also higher in the Lake Pavin ecosystem (2.3 vs. 1.9). The same was true for food web connectance, which pertains only to transfers among living compartments (1.8 vs. 1.5).

The Finn Cycling Index (FCI), showed higher recycling activity through the Lake Pavin (6%) than the Lake Aydat (3%) food web due to the higher exploitation of zoospores energetic by higher trophic levels and stronger loops. CCI, an update of the FCI index, also showed a higher recycling for Lake Pavin compared to Lake Aydat (7% vs. 4%).

All trophic level efficiencies derived from the Lindeman spine analysis were higher in Lake Pavin with the exception of trophic level III, which was more efficient in carbon transfer in Lake Aydat (66.10%) than Lake Pavin (48.33%) (**Table 6**). Trophic Level III was represented by zoospores, micro- and mesozooplankton. The global trophic efficiency (Geff) index confirmed that carbon was better transferred from the lowest to the highest trophic levels in Lake Pavin (47.09%) (**Table 6**). The Geff index is linked to grazing chain efficiency.

The Lake Pavin network exhibited a lower rate of detritivory (219 vs. 576 mgC $m^{-2}d^{-1}$) and circulation within the detrital pool (42.98 vs. 144.68 mgC $m^{-2}d^{-1}$) compared to Lake Aydat. However, the D/H ratio was higher in Lake Pavin than in Lake Aydat (**Table 6**).

DISCUSSION

PARASITES AND ALGAL BLOOMS

The planktonic food webs modeled in our study are considered to be representative of intensive algal proliferation (bloom) situations in two contrasting freshwater ecosystems. Lake Pavin is characterized by the dominance of diatoms during spring blooms while Lake Aydat shows a dominance of cyanobacteria during autumn blooms. In the classical food web version with no parasites, phytoplankton biomass produced during such proliferation events were considered to be lost by sinking (Malone, 1980; Legendre and Le Fevre, 1991; Kiorboe, 1993), and thus not able to sustain higher trophic levels. Some blooming species are even considered to be toxic, leading to reproductive failure in marine and freshwater invertebrates, interfering with zooplankton feeding (Rohrlack et al., 1999) and limiting their distribution (Ianora and Miralto, 2010). A study on the feeding behavior of

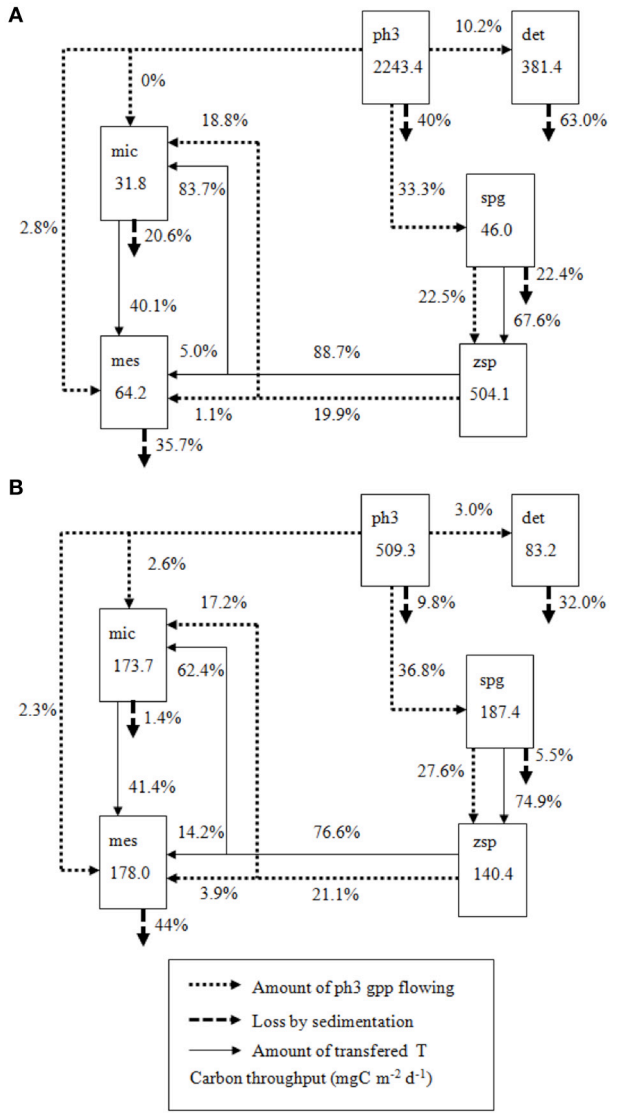

FIGURE 2 | Carbon sinking and flowing from ph3 compartment to the other compartments with highlights on carbon involved in chytrids for (A) Pavin bloom model and (B) Aydat bloom model. Bac, bacteria; Ph1, picophytoplankton; Ph2, nanophytoplankton; Ph3, microphytoplankton; Hnf, heterotrophic nanoflagellate; Mic, microzooplankton; Mes, mesozooplankton; Spg, sporangia; Zsp, zoospores; Det, detritus; Doc, dissolved organic carbon.

copepods during phytoplankton spring blooms showed that non-phytoplankton prey supported 40–71% of the copepod carbon requirement (Kobari et al., 2010).

However, it has been shown that some mesozooplankton (i.e., Cladocera) coexist with blooming filamentous cyanobacteria (Epp, 1996), and phytoplankton parasites can constitute an important food source during such situations. Indeed, fungal life stages could represent key intermediates in the pelagic food chain (Gleason et al., 2009). Chytrid zoospores were found to be efficiently grazed by mesozooplankton (Kagami et al., 2004) and could even sustain *Daphnia* growth cultured *in vitro* (Kagami et al., 2007). There has been evidence for higher instances of parasitism leading to less ecosystem reliance on detritus consumption and its related recycling (Niquil et al., 2011).

Our study focused on the algal bloom peak episodes in Lakes Pavin (from 4 to 18 April 2007) and Aydat (from September 24 to October 10 2007) and complements previous work conducted during the spring epidemic of chytrid parasites (whole spring diatom growth, from March to June 2007) in Lake Pavin (Grami et al., 2011), which found that parasitism indirectly channels primary produced carbon to grazers and that this process is an important part of the food web functioning. We were able to show the importance of parasites as trophic links, the importance of parasitic zoospores as nutritional sources able to sustain zooplankton diet, and their role in supporting upper trophic levels and recycling algal biomass.

CARBON FLOWS INVOLVED IN PARASITISM

Parasitism affected the flow of carbon during the diatom peak bloom in Lake Pavin (676.25 mgC m^{-2} d^{-1}) and the cyanobacteria peak bloom in Lake Aydat (2520 mgC m^{-2} d^{-1}). Here, total gross primary production in both lakes was higher than reported in the previous model constructed for Lake Pavin during a longer period (Grami et al., 2011; 360.54 mgC m^{-2} d^{-1}, March to June 2007). These values corroborate previous findings on the trophic status of both an oligo-mesotrophic lake (Amblard et al., 1992) and a eutrophic lake (Aleya et al., 1988). These values are consistent with gross primary production measured during a study conducted during spring phytoplankton proliferation in the north basin of the mesotrophic Japanese Lake Biwa (1639 mgC m^{-2} d^{-1}; Yoshimizu et al., 2001).

A large amount of the dominant primary producer's carbon (microphytoplankton) was involved in sporangia development (37% and 33%, respectively, in Lake Pavin and Lake Aydat); representing 28% and 29% of their total primary production. During both blooms the planktonic community was characterized by large inedible algae, high biomass and high gross primary production (*ph3* GPP). Under these circumstances, the channeling of primary production through the food web should be highly impacted by the presence of chytrids. Values of the highest possible impact of chytrids given by model simulations of different rates of parasitism on microphytoplankton tested in the Lake Biwa by Niquil et al. (2011), were around 35% of microphytoplankton GPP.

Grazers were found to be indirectly sustained by primary production through the consumption of parasitic zoospores. Indeed, a large percentage of the throughput originating mainly from sporangia was channeled to microzooplankton (76% and 83% for Lake Pavin and Lake Aydat, respectively). Values based on the mean spring model of Lake Pavin (Grami et al., 2011), were lower: only 21% of the microphytoplankton production was channeled through chytrids sustaining 38% of microzooplankton diet. Considering the higher primary production input during the bloom period, we compared our results with those obtained from a modeling study of Lake Biwa (Niquil et al., 2011). The primary production rate was around 1580 mgC m^{-2} d^{-1} and the estimated chytrid contribution to the diets of microzooplankton was only 10%; bacterial prey were the main carbon source (70% of

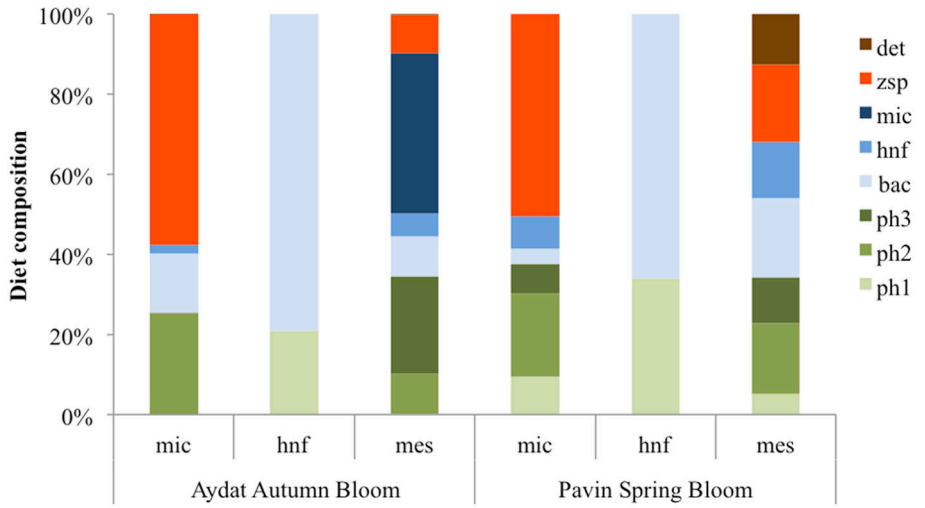

FIGURE 3 | Diet composition of each grazer for each Lake. Bac, bacteria; Ph1, picophytoplankton; Ph2, nanophytoplankton; Ph3, microphytoplankton; Hnf, heterotrophic nanoflagellate; Mic, microzooplankton; Mes, mesozooplankton; Spg, sporangia; Zsp, zoospores; Det, detritus; Doc, dissolved organic carbon.

Table 4 | Percentage of total carbon throughput loosed by sedimentation and contribution to detritus throughput of each compartment and for both Lakes.

	Pavin spring bloom		Aydat Autumn bloom	
	Loss	**det**	**Loss**	**det**
ph1	0.00	0.00	0.00	0.00
ph2	0.44	0.84	0.16	0.04
ph3	29.52	18.62	**57.74**	**60.02**
bac	0.00	2.52	0.00	11.54
hnf	0.00	15.21	0.00	2.13
mic	1.50	**23.85**	9.72	19.23
mes	**46.50**	**24.38**	6.07	6.97
zsp	0.00	10.09	0.00	0.04
spg	6.10	4.50	10.73	0.04
det	15.94	0.00	15.57	0.00

Bold indicates higher values.

their diet, Niquil et al., 2011). In a previous study, Nakano et al. (1998) reported a low ingestion rate of HNF during summertime. This confirms our hypothesis that chytrid spores could have been previously misidentified in the flagellate community and therefore have a high potential as a trophic link. During the bloom they are able to replace bacterivory and lead a higher recycling of large phytoplankton biomass.

Estimation of microbial food web efficiency (microzooplankton efficiency; 36.7% vs. 14.4%) and the microbial link (percentage of mesozooplankton demand; 53% vs. 50%), showed higher values for Lake Pavin compared to Lake Aydat. Microzooplankton efficiency, which was significantly higher for Lake Pavin, represents carbon transfer efficiency from micro- to mesozooplankton (Gaedke and Straile, 1994) calculated as microzooplankton ingestion by mesozooplankton divided by microzooplankton throughput. Based on this percentage, we could conclude that Lake Pavin, due to its higher primary production, transferred to sporangia *via* parasitism (36.8% vs. 33.25%) then to zoospores (74.86% vs. 67.6%) possessed a higher microbial food web efficiency than Lake Aydat. Comparing our models with models from the literature reveals that Lake Pavin, which is characterized by large inedible algae with high parasitism of the dominant species, has similar values to environments characterized by primary production, mainly from edible picophytoplankton (as estimated by Niquil et al., 2001 in the Takapoto atoll). Therefore, based on microzooplankton efficiency and microbial link values (**Table 7**), parasites are able to replace inedible phytoplankton, thus providing edible resources that appear to be sufficient to meet zooplankton energy requirements and sustain the carbon demand of higher trophic levels.

Parasitism could allow a better carbon transfer from primary producers to zooplankton. This pathway (producers > parasites > grazers), called the "Mycoloop" by Kagami et al. (2007), was quantified in this study during the particularly large algae proliferation. The presence of parasites allowed not only a recycling of the high input of phytoplankton biomass through an increase of energy transfer from primary producers to consumers, but also a consequent overcoming of the trophic bottleneck created by the large amount of inedible biomass in the lake.

The mycoloop may also have an impact on sedimentation rates, which are usually very important during an inedible algal bloom. When considered for the first time in a pelagic food web model, parasitism induced less carbon loss from the pelagic zone by reducing direct sedimentation of large phytoplankton species (21–10% of *ph3* GPP) and their detritus production (11–3% of their *ph3* GPP) (Pavin spring model, Grami et al., 2011). During the two bloom situations, Lake Pavin had a lower percentage of carbon loss by sedimentation from the microphytoplankton compartment (9.8% vs. 40% of microphytoplankton GPP), lower detritus production by *ph3* (3% vs. 10.2% of microphytoplankton GPP) and better carbon transfer from microphytoplankton to

Table 5 | Topological indices of pelagic food webs of Lake Pavin Spring bloom and Lake Aydat Autumn bloom; and *t* test results.

	Abv	Lake pavin	Lake aydat	Test *t p* value	Alpha 0.05
Total system throughput	TST	2625	**8889**	<0.0001	**
Average path length	APL	**2.9**	2.5	0.056	ns
Development capacity	DC	8420	**26,054**	<0.0001	**
Ascendency	A	5134	**17,287**	<0.0001	**
Relative ascendency	A/DC	61%	**66%**	0.039	*
Average mutual information	AMI	**1.96**	1.94	0.82	ns
Relative redundancy	R/DC	**47%**	45%	0.125	ns
Internal capacity	DCi	7097	**21,472**	<0.0001	**
Internal ascendency	Ai	3109	**9650**	0	**
Internal redundancy	Ri	3988	**11,821**	<0.0001	**
Internal relative ascendency	Ai/DCi	44%	**45%**	0.542	ns
Internal relative redundancy	Ri/DCi	**56%**	55%	0.5	ns

*Bold indicates higher values. *Alpha < 0.05; **Alpha < 0.01.*

Table 6 | Indices derived from the Lindeman spine.

	Pavin spring bloom	Aydat autumn bloom
TROPHIC LEVEL (TL) EFFICIENCY (%)		
1st TL	**64.82**	56.03
2nd TL	**50.18**	45.60
3rd TL	48.33	**66.10**
4th TL	**31.29**	12.90
Global trophic efficiency	**47.09**	38.42
GRAZING CHAIN EFFICIENCY (%)		
1st TL	**80.04**	64.08
2nd TL	**40.17**	29.22
3rd TL	**19.41**	19.31
4th TL	**6.07**	2.49
DETRITIVORY/HERBIVORY		
D/H	**67.44**	55.44

Bold indicates higher values.

sporangia (36.8% vs. 33.3%). Some studies observed that during a bloom 40–60% of the cells reaching the hypolimnion were still viable (Amblard and Bourdier, 1990). It would therefore appear that in the absence of parasites the majority of algal production is lost by sinking and is unavailable to support higher trophic levels. In the eutrophic Lake Aydat, the cyanobacteria production was so high that even parasitism could not help channel high rates of this carbon to higher trophic levels (i.e., zooplankton) and the carbon loss due to sedimentation of microphytoplankton still accounted for 57.7% of the total. In oligo-mesotrophic Lake Pavin, parasitism helped to reduce carbon sedimentation loss to only 9.8%, during the peak of spring diatoms, 20% of algal production was channeled to higher trophic levels and lost afterwards by sinking of the large grazers.

ECOSYSTEM EMERGENT PROPERTIES

This paper sets out, for the first time, a model that explicitly investigates the influence of parasitism on pelagic food web properties under algal bloom situations in two different lake ecosystems. Indeed, using the LIM-MCMC method, we quantified and compared the amount of carbon that reached high trophic levels *via* the mycoloop.

Past studies have investigated the impact of parasites on ecosystem properties (Huxham et al., 1995; Thompson et al., 2005; Lafferty et al., 2008; Sato et al., 2011). If high relative ascendency (A/DC) ratios reflect high degrees of organization (Ulanowicz, 1986), then, in the presence of parasites, Lake Aydat is slightly more organized than Lake Pavin (66% vs. 61%, significant difference at $P < 0.05$). However, due to redundancy, the ecosystem structure can evolve to counter the effects of external perturbations (Ulanowicz, 2003). In this case, Lake Pavin should be only slightly more resistant to stress than Lake Aydat. Ulanowicz et al. (2009) stated that the balance between the organized and non-organized parts of ecosystems increases resistance and stability; Heymans et al. (2002) suggested that redundancy should increase ecosystem resilience. If so, Lake Pavin and Lake Aydat should be equally stable and resilient.

Ascendency combines the total system activity (TST), with the organization by which the component processes are linked (AMI). It gauges how well the system is performing at processing the given medium. Chytrids were found to allow a higher fraction of the total system throughput to pass along specialized pathways (Grami et al., 2011). The degree of specialization measured by the AMI index (Ulanowicz, 1997) gave almost no difference between Lake Aydat and Lake Pavin during blooms, suggesting that both lakes have equally specialized pathways.

Baird and Ulanowicz (1993) considered the internal ascendency indices (Ai/DCi), calculated over only internal exchanges, as an aspect of a highly organized ecosystem. Given that, Lake Aydat and Lake Pavin, with almost equal Ai/DCi index, might have the same tendency to internalize their activity. Since a relevant decrease was observed for the Ai/DCi ratio in relation to the A/DC ratio for both lakes, it could point to a strong dependency of Lake Pavin and Lake Aydat on external inputs (Baird and Heymans, 1996). However, Rutledge et al. (1976) and Baird et al. (1998, 2004) considered the internal redundancy (Ri/DCi) as a measure of ecosystem stability. If so, with no significant difference between values of Ri/DCi given for Lake Pavin and Lake Aydat,

Table 7 | Comparison of some food web indicators (main contributors to PP, Microbial food web efficiency, Microbial Link and trophic efficiency at Level II) calculated for Lake Pavin and Lake Aydat and other ecosystems.

	Lake Biwa	Lake Kinneret	Takapoto atoll	English channel	Celtic sea	Aydat lake	Pavin lake
References	Niquil et al., 2011	Stone et al., 1993	Niquil et al., 2001	Vezina and Platt, 1988	Vezina and Platt, 1988	This study	This study
Main contributor to primary production (% of GPP)	phyto. > 20 μm (70%)	Non-pyrrophytes (90%)	phyto. < 3 μm (74%)	ND	ND	phyto. > 20 μm (89%)	phyto. > 20 μm (75%)
Microbial food web efficiency or microzooplankton efficiency (Mic-mes/mic throughput) (%)	10	26	38	37	46	14.40	36.70
Microbial Link as % of mes demand (mic-mes+ bac-mes/mes throughput) (%)	5.50	16	29	23	20	50	53
Trophic efficiency at Level II (%)	16	38	27	41	32	45.60	50.18

it seems that broken pathways might be equally re-established for both, which would result in the same level of stability and resilience.

Parasites are known to add links to food webs (Lafferty et al., 2008), as observed in estuarine (Lafferty et al., 2005; Thompson et al., 2005; Kuris et al., 2008) and freshwater (Kudoh and Tokahashi, 1990; Sukhdeo, 2010; Amundsen et al., 2013) ecosystems. Even if this seems logical, as for the addition of any species to a food web, this is especially true for parasites that have a free-living stage that can interact with other compartments and that may be eaten (Gross et al., 2009), such as chytrids zoospores. Parasitism also enhances the number of flows (Grami et al., 2011) and the number of compartments through which each inflow passes. Lake Pavin, with a slightly higher average path length (APL) value than Aydat (**Table 5**), has a longer chain length. This important structural property defines the number of links in a food chain and the energy transfer through its components (Jordan et al., 2003). According to Neutel et al. (2002), parasites with their complex life cycles, have more carbon loops with low interactions, which can offset the destabilizing effect of higher connectance. Lafferty et al. (2008) found that parasitism enhances connectance two fold. Chytrids in the Lake Pavin increased slightly the number of links and enhanced especially the inter-compartmental connectance compared to Lake Aydat, which represents the robustness of interactions inside an ecosystem, a measure of its organization. Robustness, indeed, is considered as a parameter linked to the stability of an ecosystem.

Cycling helps ecosystems optimize exploitation of resources by allowing a better use of energy and matter introduced into the system. In an ecosystem with low cycling (calculated by FCI), loss of carbon is thought to be higher, such as in the case of Lake Aydat compared to Lake Pavin. When perturbations occur, cycling reduces impacts to the ecosystem by acting as a buffer against large changes and can increase the ability of the ecosystem to resist changes. Then, the higher FCI in Lake Pavin increases the residence time of carbon within the ecosystem, making it more resistant to change. Another simple and effective measure of the quantitative importance of cycling in ecosystems is the comprehensive cycling index (CCI), which is one of the principal ways that ecosystem complexity can enhance stability (Allesina and Ulanowicz, 2004). CCI was also higher in the Lake Pavin ecosystem. Odum (1969) had identified the amount of cycling as one of his 24 criteria for "mature" or developed ecosystems. Wulff and Ulanowicz (1989) suggested that the increased amount of cycling is a homeostatic response of the ecosystem to stress.

CONCLUSIONS

To our knowledge, field data on pelagic ecosystem trophodynamics during bloom events with and without parasites do not yet exist. Most models use the basic tenet "zooplankton eat phytoplankton" and parasites have long been considered as playing an insignificant role in ecosystem steady-state functioning. Our models document the carbon transfer channeled by parasites from primary producers to consumers in two ecosystems dominated by inedible phytoplankton biomass and production. We provided quantitative estimates of the importance of parasitism as an indirect pathway channeling primary production contained in "inedible algae" and showed that this primary-produced carbon is efficiently conveyed to grazers and able to sustain zooplankton during inedible algal blooms, especially in the oligomesotrophic

Lake Pavin. By considering parasites, our study indicates that the ecosystem becomes more efficient and specialized, and less reliant on detritivory, which is consistent with various suggestions about the influence of parasitism on ecosystem properties. In short, parasitism on phytoplankton stabilizes the system significantly during inedible algal blooms, especially in the case of oligo-mesotrophic lakes. Ecological Network Analysis indices proved to be suitable tools for evaluating ecosystem properties linked to ecosystem stability (as resistance or resilience) in different trophic status environments. An interesting possibility would be to consider using these indices as ecological status indicators for climate change effect studies.

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Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia

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The aphelids are a small group of intracellular parasitoids of common species of eukaryotic phytoplankton with three known genera *Aphelidium, Amoeboaphelidium,* and *Pseudaphelidium,* and 10 valid species, which form along with related environmental sequences a very diversified group. The phyla Microsporidia and Cryptomycota, and the class Aphelidea have recently been considered to be a deep branch of the Holomycota lineage forming the so called the ARM-clade which is sister to the fungi. In this review we reorganize the taxonomy of ARM-clade, and establish a new superphylum the Opisthosporidia with three phyla: Aphelida phyl. nov., Cryptomycota and Microsporidia. We discuss here all aspects of aphelid investigations: history of our knowledge, life cycle peculiarities, the morphology (including the ultrastructure), molecular phylogeny, ecology, and provide a taxonomic revision of the phylum supplied with a list of species. We compare the aphelids with their nearest relatives, the species of *Rozella*, and improve the diagnosis of the phylum Cryptomycota.

Keywords: Opisthosporidia, Aphelida, Cryptomycota, Microsporidia, *Rozella,* ultrustucture, molecular phylogeny, ecology

INTRODUCTION

The aphelids are a small group of intracellular parasitoids of algae, which are currently placed in the class Aphelidea (Gromov, 2000). The class Aphelidea includes two freshwater genera, *Aphelidium* and *Amoeboaphelidium,* and a marine genus, *Pseudaphelidium.* Although for a long time members of the class Aphelidea had uncertain affinities, the phylogenetic position of this class has been recently clarified by molecular phylogenetic analyses of *Amoeboaphelidium protococcarum* (Karpov et al., 2013). The aphelids belong to the supergroup Opisthokonta, which includes multicellular animals and fungi, and a variety of unicellular organisms, which over the past decade the molecular phylogeny has been tied to each of the two following major clades (Paps et al., 2013). The Metazoa, Choanoflagellata, and Mesomycetozoea now form the Holozoa (Torruella et al., 2012), whereas nucleariid amoebae, fungi, rozellids (Cryptomycota), aphelids, and microsporidia form the Holomycota (Liu et al., 2009; Lara et al., 2010; Jones et al., 2011a; Karpov et al., 2013; Letcher et al., 2013). Recent molecular phylogeny analyses show that the class Aphelidea is sister to both Microsporidia and Cryptomycota (Karpov et al., 2013). This strongly argues in favor of the re-classification of Aphelidea at the phylum rank. All the three phyla form a separate branch sister to classical ("true") fungi, which include Dikarya (Ascomycota and Basidiomycota), paraphyletic Zygomycota, and Chytridiomycota *sensu lato* (Voigt et al., 2013).

This review focuses on the aphelids and discusses their phylogeny, life cycle, morphology, ecology, and their taxonomy. Because of their close phylogenetic relationship and life cycle similarity (in contrast with the fast-evolving Microsporidia), we often consider them in comparison with Cryptomycota. Indeed, the aphelids have a life cycle similar to that of the Cryptomycota, but are parasitoids of algae, and not of zoosporic fungi and Oomycetes as are the known species of *Rozella.*

APHELID RELATIONSHIPS: HISTORICAL SKETCH

Historical interpretations of the phylogenetic affinities of the aphelids were thoroughly discussed by Gromov (2000). Therefore, we only highlight here some of the important points. The research on aphelids began in the 19th century when the genus *Aphelidium* Zopf was first described (Zopf, 1885). 40 years later in 1925, *Amoeboaphelidium* Scherffel was described, and both these organisms were treated as the Cienkowski's "Monadinea" group, comprised of the extremely divergent "fungal animals" – organisms with a fungal-like life cycle, but having an amoeboid trophic stage (ref. in Gromov, 2000). In the 1950–1960s, the aphelids were included in the order Proteomyxida or subclass Proteomyxidia within the class Rhizopoda (Hall, 1953; Honigberg et al., 1964; Kudo, 1966). However, subsequently these protists were completely forgotten in classifications in later years (Levine et al., 1980; Karpov, 1990; Page and Siemensma, 1991; Cavalier-Smith, 1993, 1996/1997). This is

difficult to understand because during the 1960s and 1970s a number of articles were written on *Aphelidium* and *Amoeboaphelidium* by Schnepf et al. (1971) and Gromov et al. (ref. in Gromov, 2000). By the end of the last century we knew much more about the life cycles, ultrastructure and biological peculiarities of several species of aphelids. Gromov (2000) reviewed this material and established a new class Aphelidea for *Aphelidium* Zopf, 1885, *Amoeboaphelidium* Scherffel, 1925, and *Pseudaphelidium* Schweikert et Schnepf, 1996.

Until recently, the relationship between the aphelids and fungi was unclear. Cavalier-Smith (1998) suggested that the genus *Aphelidium* belongs to the opisthokonts because of their posteriorly directed uniflagellate zoospores and flat mitochondrial cristae. Gromov (2000) placed the class Aphelidea in the phylum Rhizopoda *sensu lato* on the basis of the amoeboid nature of the trophozoite stage, despite an unpublished 18S rRNA partial sequence of *Amoeboaphelidium protococcarum* which suggested a relationship with Choanozoa (Pinevich et al., 1997). Later Karpov (Adl et al., 2005) transferred the class Aphelidea into the phylum Mezomycetozoea based on the preliminary 18S rRNA molecular phylogeny of *Aphelidium*. In addition to these classifications based on the 18S rRNA marker, aphelids were placed within Ichthyosporea based on their parasitic nature (Shalchian-Tabrizi et al., 2008), and then, based on their morphology and lifestyle, as a "new" order Aphelidida, class Rozellidea, in the new subphylum Paramycia Cavalier-Smith (2013) of the phylum Choanozoa Cavalier-Smith, 1981 (Cavalier-Smith, 2013). The creation of a "new" Aphelidida order was unjustified, since that order had already been established by Gromov (2000) earlier.

Previous classifications attempts based on 18S rRNA partial gene sequences were affected by the limited resolution of this marker for the eukaryotic tree. Only recently the molecular phylogeny of *Amoeboaphelidium protococcarum* (strain x-5 CALU), based on five genes (RPB1, RPB2, 18S, 28S, and 5.8S rRNA), unambiguously showed that the aphelids branch together with Cryptomycota (*Rozella* + related environmental sequences) and microsporidia forming the ARM (Aphelidea + *Rozella* + Microsporidia) branch (Karpov et al., 2013; **Figure 1**). Letcher et al. (2013) confirmed the phylogenetic position of *Amoeboaphelidium* by isolating one more strain (FD01) of *Amoeboaphelidium protococcarum* and studying its ultrastructure and molecular phylogeny based on 18S, 5.8S, and 28S rRNA gene sequences.

The fact that the aphelids form a monophyletic group with cryptomycota/rozellids and microsporidia to the exclusion of Chytridiomycota and other fungi is also reflected in the possession of a distinctive morphological feature. According to Gromov (2000) a unique characteristic of aphelids is the intracellular trophic stage which is amoeboid and which engulfs the host cell contents. A similar stage is found in *Rozella* (Powell, 1984), but is absent in fast evolving and highly derived Microsporidia (Vávra and Lukeš, 2013). This characteristic strongly differentiates the aphelids and *Rozella* from Chytridiomycota and other fungi, and is unambiguously supported by molecular phylogeny of *Rozella allomycis* and two strains of *Amoeboaphelidium protococcarum* together with their related environmental sequences (James et al., 2013; Karpov et al., 2013; Letcher et al., 2013).

LIFE CYCLES

The complex life cycles of *Aphelidium, Amoeboaphelidium,* and *Pseudaphelidium* appear to be very similar to each other (**Figure 2**) and superficially similar to those of many species of Chytridiomycota with endobiotic development in their algal host cells (Gromov, 2000).

As an example, the *Aphelidium* opisthokont zoospore attaches to the host algae and encysts while losing its flagellum (**Figure 3A**). A cyst germinates and penetrates the host cell wall with an infection tube. The posterior vacuole appears in the cyst, enlarges, and then pushes the contents of the cyst into the interior of the host cell through the infection tube (**Figures 2** and **3A**). The parasitoid becomes the intracellular phagotrophic amoeba which engulfs the host cytoplasm with pseudopodia and transports the food into a central digestive vacuole. The parasitoid grows forming an endobiotic plasmodium with residual body while it totally consumes the cytoplasm of the host cell (**Figures 2** and **3B**). A multinucleate plasmodium is formed with a large central vacuole and a residual excretory body. The parasitoid does not form its own sporangium wall; rather it uses the host cell walls as the sporangium wall (**Figure 3C**). The mature plasmodium then divides into a number of uninucleated cells (**Figures 2** and **3C,D**). After maturation, the uniflagellated zoospores of *Aphelidium* are released from the empty host cell through the hole made previously by the infection tube and infect other algae (**Figures 2** and **3D,E**).

All known aphelids can produce multiple infections (**Figures 2** and **3**). In the case of *Amoeboaphelidium,* several amoebae grow separately in the infected alga but later fuse into a multinucleate cell, forming a single plasmodium in each infected cell (Gromov and Mamkaeva, 1968).

Sometimes giant multinucleate amoebae are released along with the uninucleate amoebae from the sporangium (Gromov and Mamkaeva, 1968). The origin of these giant amoebae is not clear, but they might result from the incomplete divisions of the mature plasmodium (Gromov and Mamkaeva, 1968). Most of these giants died after a short period of activity in culture.

Originally, amoeboid zoospores without any traces of flagella were described for *Amoeboaphelidium* (Scherffel, 1925; Gromov and Mamkaeva, 1968; Gromov, 2000). Further investigations of *Amoeboaphelidium protococcarum* (strain x-5 CALU) showed that amoebae actually have a posterior immobile pseudocilium (**Figure 4** – see discussion below). Gromov (2000) assumed that the type of propagule produced is genus specific. Indeed, the *Aphelidium* produces zoospores with a posterior flagellum (**Figures 2** and **3**), *Amoeboaphelidium* produces amoeboid zoospores with posterior pseudocilium (**Figures 2** and **4**), and *Pseudaphelidium* produces amoebae, which encyst. The cysts release one, two or, more often, four uniflagellated zoospores after germination (**Figure 2**). *Pseudaphelidium* zoospores lack the refractive globule, characteristic of *Aphelidium*.

Some aphelid species produce dormant or resting spores which are thought to be resistant to environmental extremes, and/or the result of sexual reproduction (Gromov and Mamkaeva, 1968, 1975; Letcher et al., 2013). In this case, the plasmodium ejects the residual body into the space between the cell wall of the alga and the

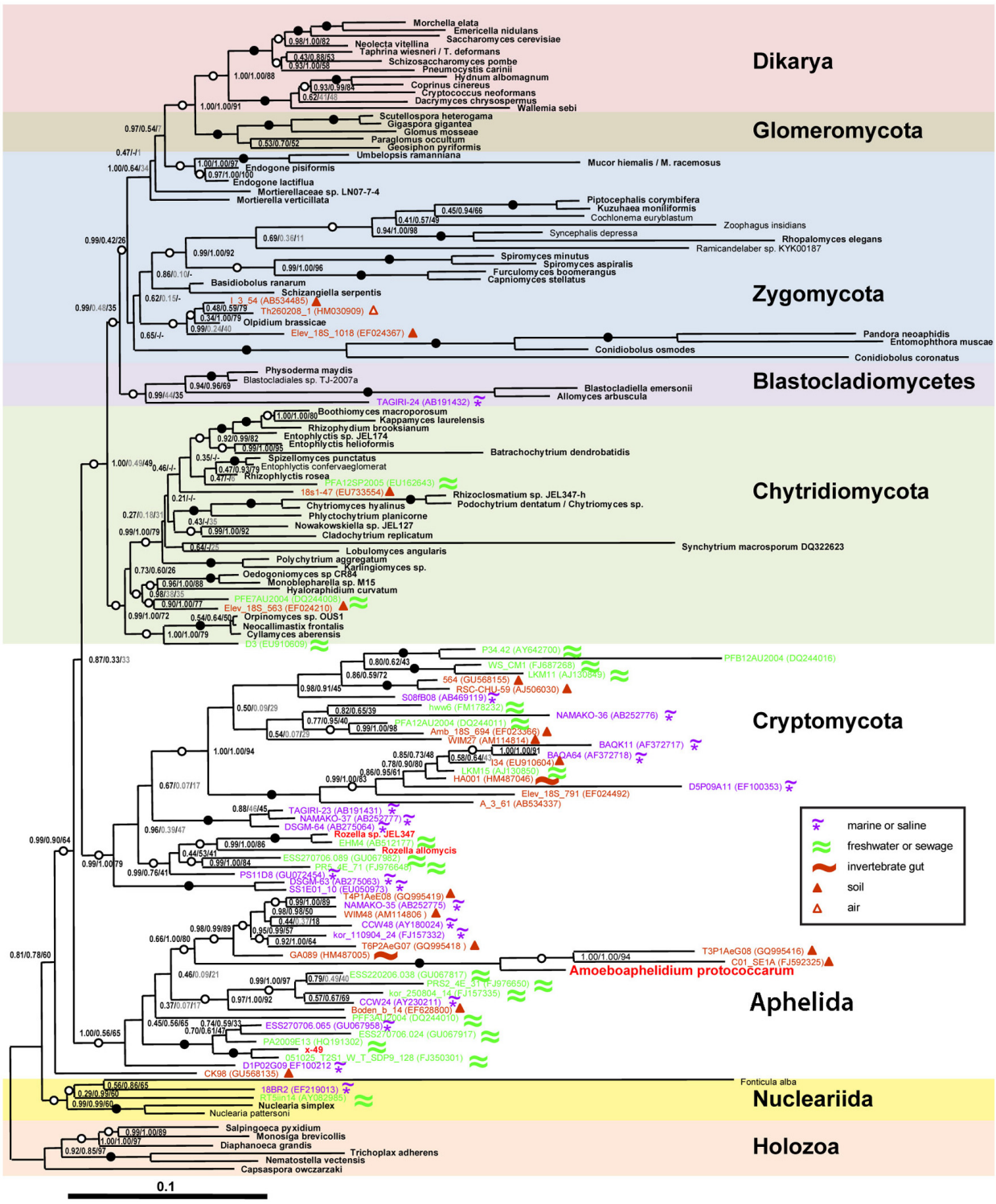

FIGURE 1 | Position of *Amoeboaphelidium protococcarum* on the tree inferred from rDNA analyses by Bayesian and ML methods (after: Karpov et al., 2013). PhyloBayes tree topology was calculated from an alignment of 144 sequences and 4,384 nucleotide characters. Node support values are given as follows: Bayesian posterior probabilities (PhyloBayes/MrBayes) followed by bootstrap values (RAxML). We used the GTR + CAT model without partition by genes for PhyloBayes calculations and the same model with partition by genes for RAxML calculations. The GTR + I + Γ_{12} model was used for MrBayes calculations. Filled circles indicate that all support values are above 98%; empty circles indicate that at least one support value is above 98%. Shown in gray are support values for clades not included in the consensus trees. Symbols in the inset indicate habitats for environmental sequences. Scale bar indicates substitutions per site.

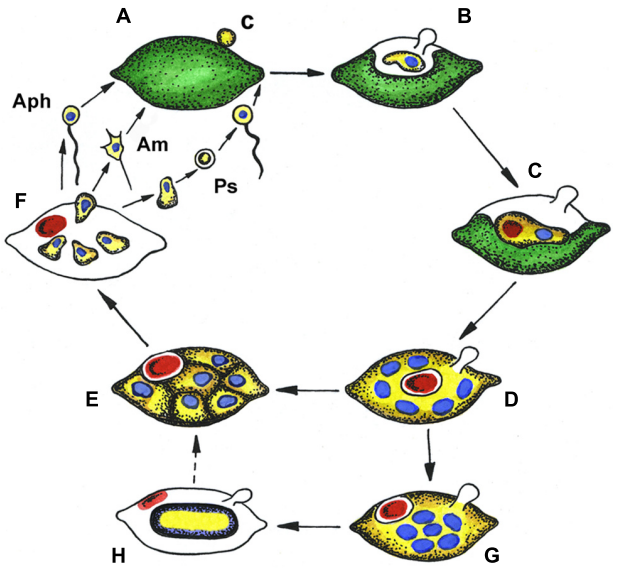

FIGURE 2 | Generalized life cycle of aphelids. *Aphelidium* (Aph), *Amoeboaphelidium* (Am) and *Pseudaphelidium* (Ps), distinguished by zoospore structure and development. **(A)** Zoospore encystment (c) on the host surface, **(B)** propagule penetration into the host, **(C)** trophic amoeba with nucleus (blue) and residual body (red) engulfs host cytoplasm, **(D)** multinuclear plasmodium (yellow) totally replaced the host, contains several nuclei (blue) and central vacuole with residual body (red), **(E)** plasmodium divides producing uninuclear cells, **(F)** mature zoospores released from the empty host cell, **(G)** precursory stage to the resting spore with nuclei in the center, **(H)** resting spore with ejected residual body. Dotted line shows conceivable way from resting spore to divided plasmodium. Colors: green, host (alga) cytoplasm; yellow, parasitoid cytoplasm; blue, nucleus; red, residual body.

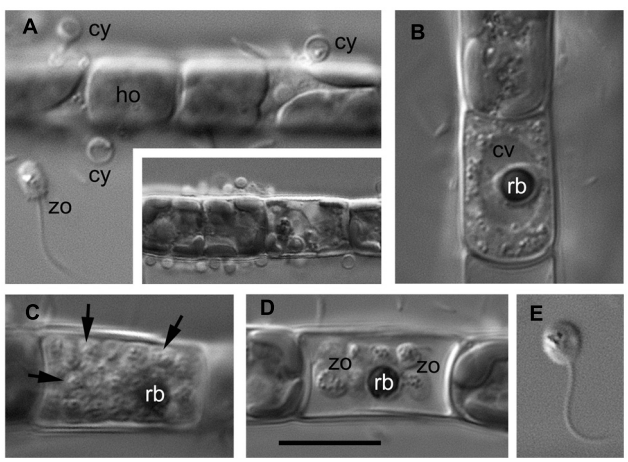

FIGURE 3 | Main stages of the life cycle of *Aphelidium* sp. parasitizing on *Tribonema gayanum* CALU- 20. Living cells observed under DIC. **(A)** Zoospore (zo) before attachment to the host (ho) and cysts (cy) on the surface of *Tribonema* filament. Insert – multiple infection at lower magnification. **(B)** Plasmodium with central vacuole (cv) and residual body (rb). **(C)** multicellular stage of parasitoid with rb at the periphery (arrows show separate cells). **(D)** mature zoospores with flagella and residual body in the empty host cell. **(E)** Free-swimming zoospore at high magnification. Scale bar: **A–D**, 10 μm, insertion in **A**, 15 μm, **E**, 8 μm.

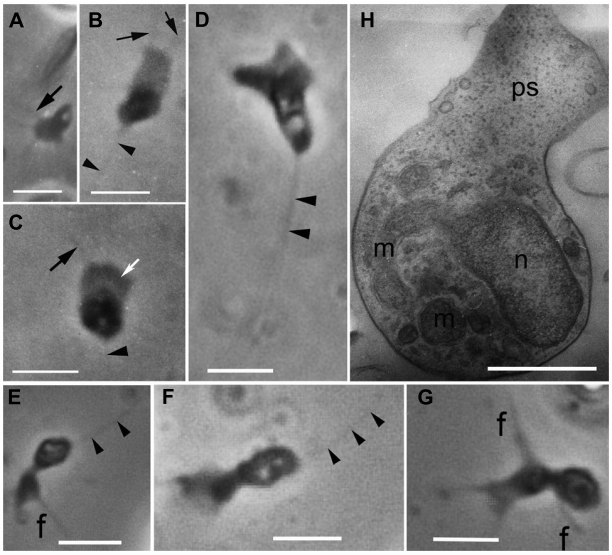

FIGURE 4 | Amoeboid zoospores of *Amoeboaphelidium protococcarum*. Living cells observed using phase contrast. **(A–C)** Strain x-1 CALU, **(D–G)** strain x-5 CALU. **(H)** Amoeba of *Amoeboaphelidium protococcarum* strain x-5 CALU on ultrathin longitudinal section. **(D–H)** After: Karpov et al. (2013). f and black arrows, filopodia often producing by broad anterior pseudopodium (ps, white arrow), m, mitochondrium, n, nucleus, arrowheads show pseudocilium. Scale bars: **A–C**, 3 μm; **D–G**, 2 μm; **H**, 1 μm.

surface of the parasitoid. The plasmodium then slightly constricts, produces a thick yellowish cell wall inside the empty host and becomes a resting cyst or dormant spore (**Figure 2**). The nuclei of the spore aggregate into the central irregular mass. During germination a spore wall becomes thin and colorless, and the plasmodium divides into several rounded cells. Further proliferation is not observed.

ULTRASTRUCTURE

MITOCHONDRION CRISTAE

The first species examined in electron microscope studies were *Amoeboaphelidium chlorellavorum* (Gromov and Mamkaeva, 1970a) and *Amoeboaphelidium protococcarum* (Gromov and Mamkaeva, 1970b). The authors fixed the samples using potassium permanganate, or osmium tetroxide (without glutaraldehyde). The latter fixative gave better results, but mitochondrial cristae appeared rounded in many sections (Gromov and Mamkaeva, 1970a,b). When *Pseudaphelidium drebesii* was fixed with glutaraldehyde the mitochondrial cristae appeared flat in cysts, but looked tubular in trophonts (Schweikert and Schnepf, 1997). These observations gave rise to the characterization of this group as having tubular cristae in the mitochondria (Gromov, 2000). In the recent studies of *Aphelidium,* the mitochondrion had lamellar cristae in zoospores and tubular cristae in cysts (Gromov and Mamkaeva, 1975). The amoebae of *Amoeboaphelidium* also have mitochondria with lamellar cristae (Karpov et al., 2013; Letcher et al., 2013). Altogether, these observations of the mitochondrion ultrastructure suggest that the shape of the mitochondrial cristae is variable in the aphelids.

FLAGELLATED ZOOSPORES

The flagellated zoospore structure was only described for *Aphelidium* (Gromov and Mamkaeva, 1975). It has one posteriorly directed acronematic flagellum with 9 + 2 axoneme, and few short filopodia at the anterior end, used for cell attachment. The nucleus is located in the anterior part of the cell. A dictyosome lies between the nucleus and the flagellar kinetosome and some mitochondria with lamellate cristae and small lipid droplets are situated around the nucleus. Ribosomes are scattered in the cytoplasm, which usually has few ER cisternae. Unfortunately, the most informative and phylogenetically important flagellar apparatus has not been studied in detail. A short centriole lies at different angles to the kinetosome from nearly parallel (Gromov and Mamkaeva, 1975) to orthogonal (Karpov, in preparation), and both are connected to each other by a rather broad fibrillar bridge. Both the amoeboid and flagellated zoospores of aphelids are uninucleated and have, in addition to the mitochondria, a small microbody with granular contents associated with the nucleus, and several lipid globules spread throughout the cytoplasm. Ribosomal aggregation is absent.

PLASMODIUM

The ultrastructure of the plasmodium of *Amoeboaphelidium* species does not differ significantly from that of *Aphelidium chlorococcarum* (Gromov and Mamkaeva, 1970a,b), and *Pseudaphelidium* (Schweikert and Schnepf, 1997). The multicellular stage in plasmodium development was shown for *Aphelidium* (Gromov and Mamkaeva, 1975), *Pseudaphelidium* (Schweikert and Schnepf, 1997), and *Amoeboaphelidium* (Letcher et al., 2013).

PENETRATION APPARATUS

Aphelids have a peculiar penetration apparatus, similar in some respects to that of microsporidia. After zoospore attachment its pseudopodium grows along the host surface seeking a hole or gap in the wall. This was shown for *Aphelidium* (Gromov and Mamkaeva, 1975) and *Pseudaphelidium* (Schweikert and Schnepf, 1997). If the pseudopodium does not find a point of entry into the host, the infection fails. It seems the penetration tube grows around the pseudopodium. After tube penetration in the host, the posterior vacuole enlarges and pushes the parasitoid out of the cyst through the infection tube into the host cell (Gromov, 2000; Karpov et al., 2013). The mechanism of injection needs the cyst wall to generate the pressure inside the cyst for cell migration. In *Pseudaphelidium* the cyst wall is thicker than in other genera, and, unlike other aphelids, a special inverted tube is present in the cyst (Schweikert and Schnepf, 1997). The proximal part of the tube is fixed at a certain place (faced to the host surface) of the cyst similar to that of the anchoring disk complex in the spore of microsporidia. This tube everts during invasion penetrating the host cell wall. In both these respects the tube is more similar to the injection apparatus of microsporidia, than to that of other aphelids. The injection apparatus of microsporidia consists of the injection tube, the polaroplast (membrane storage organelle) and a posterior vacuole (a pressure-building organelle; Vávra and Lukeš, 2013). The precise mechanism of spore extrusion is still unclear (Vávra and Lukeš, 2013).

AMOEBOID ZOOSPORES

The better studied *Amoeboaphelidium* has two forms of amoebae (aplanospores): so called "radiosa" forms (floating amoebae detached from the substrate), and freely moving amoebae. The floating amoebae are normally spherical and have radial filopodia independently of the shape of the moving morphotype. The moving amoebae are very characteristic of *Amoeboaphelidium protococcarum*: they are subdivided into two nearly equal parts: the posterior which contains the nucleus and other organelles, and the anterior which is made up of the broad and flat pseudopodium producing several thin filopodia (subfilopodia; Karpov et al., 2013; Letcher et al., 2013; **Figure 4H**). The anterior part does not contain any organelles, just a hyaloplasm. The posterior end bears a pseudocilium, which is clearly visible in both the old photographs of x-1 CALU (**Figures 4A–C**), and the recent photographs of x-5 CALU (**Figures 4D–F**). The pseudocilium contains 2–3 microtubules, at least, and seems to represent the rudimentary flagellum (Karpov et al., 2013).

Since the type strain of *Amoeboaphelidium protococcarum* (x-1 CALU) has a pseudocilium, the description for this species has been corrected (see Taxonomy). Although Letcher et al. (2013) paid particular attention to the presence/absence of pseudocilium in FD01, the authors seem to have overlooked this tiny structure, which may have been masked by the very dense cytoplasm. In some of their figures (Figures 5G,H in Letcher et al., 2013), however, the oblique profile of a microtubule is clearly visible. Although the authors marked this microtubule as the filaments, the distance between these "filaments" is precisely 25 nm – the diameter of microtubule. This suggests that the amoeboid zoospore of FD01 also has a pseudocilium.

Zoospores of the strains x-1 and x-5 have a nucleus of peculiar convex-concave shape with the microbody in the invagination. The nucleus in FD01 is more spherical and of convex-flat shape, but is also associated with the microbody. These morphological differences seem not to be significant. The organisms of all three strains (x-1, x-5 CALU, and FD01) are morphologically very similar to each other in the following ways: amoebae have the same dimensions (2–4 μm), moving morphotype, an outer and internal morphology; they infect the green chlorococcous alga *Scenedesmus*; and their life cycle contains all the stages described for *Amoeboaphelidium protococcarum*. Thus, by these criteria they should all certainly belong to the same species, *Amoeboaphelidium protococcarum*.

MORPHOLOGICAL IDENTITY VS. GENETIC HETEROGENEITY

While morphologically similar, the two strains of *Amoeboaphelidium protococcarum* FD01 from Texas (USA) and x-5 from the Russian Far East exhibited a low similarity in rRNA gene sequences, suggesting a significant phylogenetic distance. Indeed, the similarities were of 86, 84, and 78% for the 18S, the 5.8S, and the 28S rRNA genes respectively (Letcher et al., 2013). For the majority of protists, such differences would correspond to genus level differences at least. For instance, the genus level is placed at 6–10% dissimilarity in 18S rRNA genes for bicosoecids (Kim et al., 2010). It is uncertain whether such genetic distances are normal intraspecific variations for the aphelids, or not. We suggest at this stage of study that both strains should be retained in the genus

Amoeboaphelidium, but they might actually belong to different species.

Strain diversity in *Amoeboaphelidium protococcarum* was noticed earlier. Pinevich et al. (1997) compared approximate sizes and numbers of chromosomes in strains x-1 and x-5 CALU using pulsed-field gel electrophoresis (PFGE). They wrote about "similar, though not identical numbers and sizes of individual chDNAs" and "one can conclude that there is a close relatedness between x-1 and x-5" (Pinevich et al., 1997, p. 125). Thus, the number and size of chromosomes in two morphologically identical strains (x-1 and x-5 CALU) of *Amoeboaphelidium protococcarum* differ. These data show the occurrence of cryptic diversity and the importance of genetic studies of different strains within the same aphelid morphospecies. The genetic heterogeneity of morphologically indistinguishable strains is usual for some protists, e.g., syngene of ciliates (Lynn, 2008). However, further study may reveal the ultrastructural differences, which are still poorly known for aphelids.

THE COMPARISON BETWEEN APHELIDS AND ROZELLIDS

Rozella allomycis, like the aphelids, also has endobiotic development and does not produce its own sporangium wall. The more important common characteristic is the ability of trophonts to phagocytose. This fact clearly separates the Aphelidea and Cryptomycota from the fungi and unambiguously supports the molecular phylogeny of both groups. The rozellids produce flagellated zoospores, and have zoosporic fungi and Oomycetes as their hosts, rather than algae, as do the aphelids (see details in Ecology of Aphelids in Comparison with *Rozella*).

The kinetid structure of the zoospores produced by *Rozella* is better known and differs essentially from that of *Aphelidium*. The flagellum in *Rozella* emerges from the bottom of a deep invagination at the cell's posterior (Held, 1975). The flagellar kinetosome is long and has two prominent rhizoplasts connecting the kinetosome to the mitochondrion (Held, 1975). *Aphelidium* has a flagellum emerging from the flat surface of the zoospore and a relatively short kinetosome without the roots (Gromov and Mamkaeva, 1975). In *Rozella* the centriole lies at an angle of 45° to the kinetosome (Held, 1975). Obvious peculiarities of kinetid in *Rozella* zoospores confirm its genetic difference from the aphelids, but the detailed reconstruction of *Aphelidium* kinetid is necessary.

Rozella zoospores have special endoplasmic reticulum (ER) cisterns closely associated to the nucleus (Held, 1975) reminding the inverted tube of *Pseudaphelidium*. No such cisterns or other traces of such tubes were found in *Aphelidium* (Gromov and Mamkaeva, 1975; Karpov, in preparation) and *Amoeboaphelidium* (Gromov and Mamkaeva, 1968, 1970b; Letcher et al., 2013). Thus, from the morphological perspective, the two latter genera appear to be at a greater distance from microsporidia than *Pseudaphelidium* and *Rozella.* At the same time, the *Aphelidium* might have retained ancestral features of the ARM branch, having a simpler life cycle with less complex uniflagellate zoospores.

The cyst wall of both *P. drebesii* and *Aphelidium chlorococcarum* stains with calcofluor white indicating the presence of chitin (Schweikert and Schnepf, 1997; Gromov, 2000). The cyst wall of *Rozella* is also composed of chitin (James and Berbee, 2012; James et al., 2013). The occurrence of chitin, and the presence of four chitin synthetase genes in *Rozella* contradicts former descriptions of Cryptomycota claiming that they have "cysts without a chitin/cellulose cell wall" (Jones et al., 2011b).

The presence of chitin cell walls and chitin synthetase genes in the whole ARM clade strongly suggests that the common ancestor of the fungi and ARM already possessed fungal-specific chitin biosynthesis.

ECOLOGY OF APHELIDS IN COMPARISON WITH *ROZELLA*

All known species of *Rozella* and the aphelids are obligate parasitoids (biotrophs) and must be grown in culture with their hosts (Held, 1981; Gromov, 2000; Gleason et al., 2012). Therefore, their ecology cannot be disentangled from that of their hosts. One important ecological difference is that the hosts for *Rozella* species are zoosporic fungi and Oomycetes (heterotrophic stramenopiles), while aphelid genera have a wide variety of phytoplankton species as hosts. According to Held (1981) the hosts for the class Rozellidae are placed into four phyla of zoosporic true fungi and fungal-like organisms: Chytridiomycota, Blastocladiomycota, Monoblepharomycota, and Oomycota (Saprolegnialean and Peronosporalean galaxies). As previously stated, Held (1981) briefly speculated that some phytoplankton species might be *Rozella* hosts as well, but did not consider this possibility further. Other undescribed cryptomycotes attack diatom algae, as was shown by FISH (Jones et al., 2011a). According to Gromov (2000) the hosts for the class Aphelidae belong to the phyla Chlorophyta, Xanthophyta, and Bacillariophyta (**Table 1**). The most common hosts for the aphelids are found among the chlorococcous algae, and these host-parasite relationships are often genus specific (Fott, 1957, Gromov and Mamkaeva, 1966, 1968). A xanthophyte, *Tribonema gayanum* Pasher, is the most commonly reported host for aphelids (Gromov, 1972; Gromov et al., 2002), and this species is normally used to support the cultures of these parasitoids. In nature the aphelids prefer the eutrophic water basins, where they live on planktonic algae, epiphytic algae on aquatic plants, and soil surface near the temporary and permanent water reservoirs. Mamkaeva et al. (1974) showed that *Amoeboaphelidium protococcarum* regularly occurs, sometimes in high densities, in some ponds of the Kaliningrad region of Russia. In the Ribinskoye reservoir (Yaroslavl district, Russia) its density is relatively low, and has so far never been found in sphagnum bogs. At the same time this organism has been found in 6% of samples from water basins of the Soviet Union (Mamkaeva et al., 1974). The density of parasitoid population varies significantly in different water bodies. Gromov et al. (2002) investigated the distribution of *Aphelidium* and the very common chytridiomycete, *Rhizophydium*, in 10 stations in the Ladoga lake and adjacent water bodies in August 2000, and found *Aphelidium* in 6 and *Rhizophydium* in 10 stations. The number of infectious units (zoospores or infected algal cells) for *Aphelidium* varied from 0.01 to 0.92 per 300 ml of water, while for *Rhizophydium* it was from 0.1 to 1.6. This suggests that the infection level by *Aphelidium* is comparable with that of the most common freshwater representative of Chytridiomycota. Mohamed and Martiny (2011) investigated the fungal diversity along an estuarine salinity gradient in Rhode Island by sequencing a large set of environmental 18S rRNA genes. Aphelid

Table 1 | Species of aphelids and their hosts.

Parasitoid		Host	
Genus	**Species**	**Genus and species**	**Phylum**
Aphelidium	*deformans* Zopf, 1885	*Coleochaeta* sp.	Chlorophyta
	melosirae Scherffel, 1925	*Melosira varians*	Bacillariophyta
	tribonemae Scherffel, 1925	*Tribonema gayanum*, *Botridiopsis intercedens*	Xanthophyta
	chlorococcarum Fott, 1957	*Scenedesmus armatus,* other chlorococcous algae	Chlorophyta
	chlorococcarum f. *majus* Gromov et Mamkaeva, 1970	Chlorococcous algae	Chlorophyta
Amoeboaphelidium	*achnanthides* Scherffel, 1925	*Achnanthes* sp.	Bacillariophyta
	protococcarum Gromov et Mamkaeva, 1968. emend. Karpov	*Scenedesmus obliquus, Scenedesmus dimorphus, Scenedesmus minutum, Chlorococcum* sp., other chlorococcous algae	Chlorophyta
	chlorellavorum Gromov et Mamkaeva, 1968	*Chlorella* sp.	Chlorophyta
	radiatum Gromov et Mamkaeva, 1969	*Kirchniriella* sp., *Ankistrodesmus* sp.	Chlorophyta
Pseudaphelidium	*drebesii* Schweikert et Schnepf, 1996	*Thalassiosira punctigera,* other diatoms	Bacillariophyta

Data were taken from Gromov (2000); Karpov et al. (2013), and Letcher et al. (2013).

plus rozellid sequences represented 9% of the 1095 "fungal" clones obtained, and around 25% of the phylotypes (28 out of 104). Aphelid and rozellid sequences were more abundant in freshwater marsh samples (12%), than in brackish (8%) or salt marshes (5%). The relative amount of aphelid and rozellid sequences and phylotypes was almost the same in the different libraries from Rhode Island marshes (Mohamed and Martiny, 2011), whereas normally there is a significant excess of rozellids over the aphelids in environmental samples.

In the producer-based food webs *Rozella* species are secondary consumers while aphelids are primary consumers. It is likely that both *Rozella* and aphelids can play roles in regulating the size, composition and dynamics of populations of zoosporic true fungi and Oomycetes (heterotrophic stramenopiles) and phytoplankton. Species of *Rozella* and aphelids are common parasitoids and therefore are likely to be factors which determine ecosystem complexity, although quantitative data on host-parasitoid dynamics are not yet available. Also, it is likely that species of *Rozella* can regulate the population sizes of zoosporic true fungi and Oomycetes in detritus-based food webs. Without empirical data the impact of their roles in regulation of host populations remains unknown. Preliminary data suggest that some of these parasitoids are highly virulent, and that the cytoplasm of the host appears to be converted efficiently into the cytoplasm of the parasitoid (Held, 1981; Gromov, 2000).

There is evidence that virulence of different genotypes of parasitoids and sensitivity of different genotypes of hosts to infection are variable in host-parasitoid interactions in both the Cryptomycota and the Aphelidea. The host range for *R. allomycis* has been tested in the laboratory and appears to be very narrow, with only a few genotypes of *Allomyces* being susceptible to infection (Held, 1981). Gromov and Mamkaeva (1968) measured the susceptibility of 226 different strains of green and yellow-green algae to infection by four isolates of *Amoeboaphelidium* (x-1 through x-4 CALU). Some cultures were susceptible to infection by strains x-1, and x-4 (*Amoeboaphelidium protococcarum*), while others were resistant. The strain x-2 (*Amoeboaphelidium chlorellavorum*) infected only the *Chlorella* strains.

As previously stated, the motile propagules produced by species of *Rozella* and aphelids are different. All known *Rozella* species produce uniflagellate zoospores. Aphelids can produce either uniflagellate zoospores, amoebae without flagella or amoebae with flagella reduced in size (**Figure 2**). Zoospores are adapted for swimming in water while amoebae (even with a posterior immobile pseudocilium) are adapted to crawl on surfaces (Gleason and Lilje, 2009; Karpov et al., 2013). Interestingly, *Aphelidium melosirae* and *Aphelidium tribonemae* zoospores, despite having normal flagella, have been observed to crawl like amoebae (Gromov, 1972). They produce short lobopodia (*Aphelidium melosirae*), or filopodia (*Aphelidium tribonemae*), and crawl using pseudopodia while the immobile flagellum trails behind the zoospore.

Thus, the aphelids retained the amoeboid nature in all three genera not only at trophic stage like *Rozella*, but also in propagules, which agrees with their more basal position in the molecular phylogenetic tree (**Figure 5**).

As previously stated both *Rozella* and the aphelid genera use the host cell wall as a zoosporangium. Therefore, these endobiotic species are likely to use less energy in reproduction than epibiotic species since they do not produce an extra structure for zoospore release. Since both groups have small thalli and most of the cytoplasm goes into the zoospores, reproduction is energy efficient. Therefore, a relatively high percentage of energy is likely to be transferred between trophic levels. Species in both groups are at the second or third trophic level as explained above so that bottom-up effects would impact on the entire food web above

producers or primary consumers. Because of their small sizes (Held, 1981; Gromov, 2000) zoospores produced by *Rozella* and aphelids are likely to be easily eaten by filter feeding zooplankton and metazoans at the third or fourth trophic level (Gleason et al., 2008). At least one uncultured aphelid species has been detected in fecal pellets passed through the intestinal tract of detritus-feeding freshwater amphipod *Gammarus tigrinus* from Canada (Sridhar et al., 2011). Rozellids found in the feces of studied species of amphipods accounted for 21 out of the 74 "fungal" clones in the same analysis and clustered in 10 phylotypes (Sridhar et al., 2011). In this way the nutrients of the host plankton are recycled by the microbial loop while they remain in the euphotic zones.

TAXONOMY

Presently, the Cryptomycota has a rank of phylum (Jones et al., 2011b), as has its sister group the Microsporidia in the ARM clade. But both phyla are sisters to the class Aphelidea (Karpov et al., 2013; **Figure 5**). Therefore, it is logical to change the class Aphelidea to a phylum – taxon of the same rank as Cryptomycota and Microsporidia. At the same time, the whole ARM branch is sister to all the Fungi *sensu lato*, and are not true Fungi. Thus, we now amend the taxonomy of the ARM-clade, and establish a new superphylum the Opisthosporidia with three phyla: Aphelida phyl. nov., Cryptomycota, and Microsporidia. This proposal is based on molecular phylogeny, morphological, ultrastructural and ecological characteristics of all three phyla discussed in this review.

OPISTHOKONTA Cavalier-Smith, 1987

The Opisthokonta is divided into Holomycota and Holozoa, a division based solely on molecular characteristics. Cryptomycota and the whole ARM-clade (Opisthosporidia) are not true fungi (**Figure 5**), as has also been noted by Cavalier-Smith (2013). Thus, along with nucleariids, one more branch has appeared at the Holomycota/Holozoa border. Given that more and more deep branches are appearing at this border, what has until recently appeared to be a clear distinction may become unstable, particularly with the acquisition of molecular data for pompholyxophryids, other rotospherids, and yet-unsampled potential Opisthokonta.

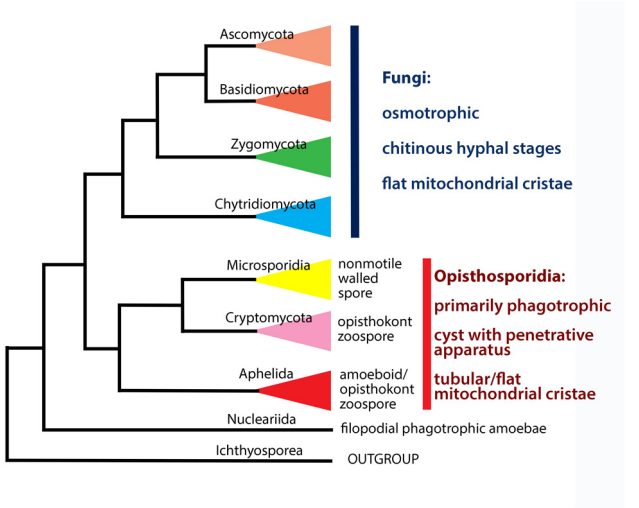

FIGURE 5 | Phylogenetic and taxonomic summary of early diverged groups of Holomycota.

OPISTHOSPORIDIA KARPOV, ALEOSHIN ET MIKHAILOV SUPERPHYLUM nov.

Opisthokont intracellular parasites/parasitoids with amoeboid vegetative stage. Invasive spores/cysts with chitin cell wall and specialized apparatus for penetration into host cell (penetration tube; posterior vacuole). If present, zoospores with filopodia and/or one posteriorly directed whiplash flagellum (functional or rudimentary). Phagotrophic or osmotrophic.

Etymology

Named by word combination of Opisthokont and sporae, making reference to the specialized penetration apparatus of the spore (in Microsporidia) or cyst (in two other phyla) characteristic for all three phyla Aphelida, Cryptomycota, and Microsporidia.

Superphylum includes three phyla (**Figure 5**): Microsporidia, Cryptomycota and Aphelida phyl. nov.

The phylum Microsporidia is well known and has a good description (Issi and Voronin, 2007; Williams and Keeling, 2011; Vávra and Lukeš, 2013). Microsporidia are protistan parasites of animals (predominantly insects and crustaceans) and rarely infect protists. Microsporidia have been known since 1882 by Balbiani and at present they account for 1300 to 1500 species, distributed in 187 genera. They are obligate intracellular parasites with a relatively uniform life cycle: a germinating spore injects the spore contents (sporoplasm) into the host cell by means of an explosively evaginable "injection tube" (polar tube or polar filament). The sporoplasm grows into cells called meronts, which divide into daughter meronts. The trophic stage is extremely simplified: it has a reduced genome, reduced ribosomes, poorly developed internal membrane system, lacks canonical dictyosomes, lost peroxisomes, and mitochondria are reduced to mitosomes which are unable to produce ATP. Microsporidia have developed a unique capacity to get ATP directly from the host cell, and became "energy parasites." The meronts progressively fill the cytoplasm of the host cell, and then produce the chitin cell wall on their surface becoming the sporonts and then sporoblasts. Each sporoblast matures into a complex infective spore equipped with an injection apparatus. The infective spore is a dispersal stage, which can survive in the environment.

It was considered, that the presence of the injection apparatus in the spore is an autapomorphic character that sharply delineates microsporidia (Vávra and Lukeš, 2013). But it can be suggested, that the rozellids (Williams and Keeling, 2011) and aphelids, in the frame of the same phylogenetic lineage, have retained the primitive injection apparatus, which is homologous to the injection apparatus of microsporidia.

The description of the phylum Cryptomycota Jones et Richards, 2011 needs to be modified. The following description: "Fungi unicellular, zoospores single-celled with a single microtubular

flagellum, and cysts without a chitin/cellulose cell wall. Forming epibiotic associations" (Jones et al., 2011b) contains many inaccuracies and certainly does not correspond to the genus *Rozella*, which is the only described genus (with approximately 20 valid species) in this phylum.

(1) "Fungi unicellular" – they are not fungi – it is a group of Opisthokonta, sister to Fungi.
(2) "Microtubular flagellum" – not a good word combination, as non-microtubular flagellum is unknown.
(3) "Cysts without a chitin/cellulose cell wall" – it is now known that the cyst of *R. allomycis* has a chitin cell wall and that this species contains four chitin synthetase genes (James and Berbee, 2012; James et al., 2013).
(4) "Forming epibiotic associations" – what are these associations? *Rozella* has cyst attached to the host surface, but does not live there, so is not epibiotic, rather it develops as an endobiont.

We propose that further improvements in the description of the phylum are necessary.

PHYLUM CRYPTOMYCOTA (Jones et Richards, 2011), EMEND. KARPOV ET ALEOSHIN

Opisthokont intracellular parasitoids, predominantly of true fungi, Oomycetes (heterotrophic stramenopiles), and diatom algae with endobiotic phagotrophic amoeboid vegetative stage. Invasive cyst with short or long infective tube of penetration apparatus. Zoospore with posterior functional flagellum.

At present, the diversity of Cryptomycota, shown by environmental sequences, is really huge (Lara et al., 2010; Jones et al., 2011a,b; Mohamed and Martiny, 2011; James et al., 2013; Karpov et al., 2013). They are found in marine, brackish and fresh waters, infect not only the fungi, like *Rozella*, but also some algae (Jones et al., 2011a). Unfortunately, the lack of clear borders for this phylum leads to the overestimation of their diversity. In the 18S rRNA gene phylogenetic trees reconstructed in the absense of aphelids (their environmental sequences were identified as the aphelids in 2013 only) and microsporidia (because of too long branches for this gene), any sequences lying between true fungi and *Nuclearia* were deemed Cryptomycota. Even in the presence of aphelids and microsporidia in the tree, these three independent branches were called Cryptomycota (Letcher et al., 2013) instead of ARM-clade.

In any case, a really broad divergence of these protists, which might be even wider than in the aphelids, suggests that we cannot exclude their parasitism on algae, and, perhaps, their saprotrophic mode of life. For further clarification we need more studies on the real organisms, to complement the molecular work that is being done.

PHYLUM APHELIDA KARPOV, ALEOSHIN ET MIKHAILOV PHYLUM nov.

Opisthokont intracellular parasitoids of algae with phagotrophic amoeboid vegetative stage. Invasive cyst with short infective tube of penetration apparatus. Zoospore with pseudopodia and/or posteriorly directed functional or rudimentary flagellum.

Rozella and aphelids are morphologically similar to each other. But the genetic distance between them is very large, what we can certainly say now having sequenced genomes of *R. allomycis* (James et al., 2013) and multigene data of *Amoeboaphelidium protococcarum* (unpublished). Moreover, each species is well nested within the two large groups defined by the environmental sequences (**Figure 1**).

CLASS APHELIDEA GROMOV, 2000

Amoeboid endobiotic parasitoids of algae. Dispersal stages in the life cycle, zoospores or amoebae, attach to a new host cell and encyst. Amoeboid body penetrates into the host's cell through a cyst stalk. The intracellular amoeba engulfs the contents of the host's cell, forming food vacuoles which transport the food into the central digestive vacuole. An excretory body is formed in the digestive vacuole. The amoeboid trophont grows into a plasmodium, which totally replaces the cytoplasm of a host cell; the multinuclear plasmodium divides into uninuclear amoeboid cells or uniflagellated zoospores. No specialized sporangium cell wall is formed by the parasitoid around the sporangium. Some species form intracellular resting spores.

Order Aphelidida Gromov, 2000. Diagnosis coincides with that of the class.

New order with the same name Aphelidida, proposed by Cavalier-Smith (2013) is not valid.

Family Aphelididae Gromov, 2000. Diagnosis coincides with that of the class.

REVIEWED DIAGNOSES OF APHELID GENERA AND SPECIES

APHELIDIUM (Zopf, 1885) GROMOV, 2000

Parasitoids of various species of algae. Forms rounded to oval zoospores, able to produce pseudopodia, with one posteriorly directed whiplash acronematic flagellum and one or several lipid globules (refractile granules). Vegetative development as described for the class. Resting spores rounded to oval with a thick smooth cell wall, and without an excretory body which is ejected from the spore before spore wall synthesis.

Type species of the genus Aphelidium deformans Zopf, 1885.

Aphelidium deformans Zopf, 1885. Parasitoid of a green alga *Coleochaeta*. Infected host cell is deformed, becoming abnormally large (up to 10 times vs. normal) with thickened cell wall. Zoospores 2–3 μm in diameter. Resting spores rounded to oval, 12–30 μm in diameter, with a large lipid granule.

Aphelidium melosirae Scherffel, 1925. Parasitoid of the diatom alga *Melosira varians* Ag. Zoospores oval, 4 × 6 μm, with several refractive granules. Flagellum is about 10 μm long. Zoospores are slightly amoeboid, can produce short lobopodia and move like amoebae having an immotile flagellum. Resting spores 12–14 × 10 μm.

Aphelidium tribonemae Scherffel, 1925. Parasitoid of a yellow-green alga *Tribonema*. Zoospores 2–3 μm in diameter, flagellum is about 7 μm long with long (5 μm) acronema. Zoospores can produce filopodia and move like amoebae with an immotile flagellum. The development of *Aphelidium tribonemae* was observed in *Tribonema gayanum* Pasch. and *Botridiopsis intercedens* Visch. et Pasch.

Aphelidium chlorococcarum Fott, 1957. Parasitoid of chlorococcous algae. Zoospores 1.5–2.0 μm in diameter. Flagellum about

8 μm long. Resting spores ellipsoid, 7.0 × 5.0–6.5 μm. Parasitoid ultrastructure from mass culture of *Scenedesmus armatus* Chod. was investigated by Schnepf et al. (1971).

Aphelidium chlorococcarum forma *majus* Gromov et Mamkaeva, 1970. Zoospores 2.0–3.0 μm in diameter, flagellum about 14 μm long. The ultrastructure of zoospores and vegetative stages investigated by Gromov and Mamkaeva (1975).

A. lacerans Bruyne, 1890 and *A. chaetophorae* Scherffel, 1925 do not correspond to the diagnosis of the genus (Gromov, 2000).

AMOEBOAPHELIDIUM (Scherffel, 1925) GROMOV, 2000, EMEND. KARPOV

Parasitoids of various species of algae. Amoeboid zoospores, with or without posterior pseudocilium, forming flat hyaline pseudopodium with subfilopodia, or filopodia of different length. Vegetative development as described for the class. Resting spores rounded to oval, with a thick cell wall.

Type species of the genus Amoeboaphelidium achnanthides Scherffel, 1925.

Amoeboaphelidium achnanthides Scherffel, 1925. Parasitoid of the diatom *Achnanthes*, amoebae about 2 μm long.

Amoeboaphelidium protococcarum Gromov et Mamkaeva, 1968, emend. Karpov. Parasitoid of chlorococcous algae, strains differ by the possible hosts. Amoebae 2.0–4.0 μm in diameter with posterior pseudocilium 7 μm long. Resting spores oval, 4–6 × 5–7 μm.

Type strains: x-1, x-4 CALU (Gromov and Mamkaeva, 1968).

Amoeboaphelidium chlorellavorum Gromov et Mamkaeva, 1968. Parasitoid of some species of the green alga *Chlorella*. Amoebae about 1 μm in diameter, extracellular cysts without a discernible stalk.

Type strain: x-2 CALU.

Amoeboaphelidium radiatum Gromov et Mamkaeva, 1969. Parasitoid of the chloroccous algae *Kirchniriella* and *Ankistrodesmus*. Amoebae 1–3 μm in diameter with limited motility, have very thin and long filopodia (10–12 μm). Development on the surface of solid culture media not observed.

Type strain: x-3 CALU.

PSEUDAPHELIDIUM Schweikert et Schnepf, 1996

Zoospores colorless, lacking a noticeable refractive granule, with one posteriorly directed whiplash flagellum with an acroneme. The body of the parasitoid penetrates the host's cell with a special infection tube everting from the cyst. Vegetative development as described for the class. Plasmodium divides into amoeboid cells, which encyst being released from sporangium. New opisthokont zoospores leave the cysts.

Type species of the genus Pseudoaphelidium drebesii Schweikert et Schnepf, 1996.

P. drebesii Schweikert et Schnepf, 1996. Zoospores 5 μm long and 3 μm wide, flagellum 15 μm long. By the end of the development plasmodium forms a hollow sphere. It divides into rounded cells, from which amoeboid cells with very limited motility are formed. Amoebae encyst (cyst diameter 4–6 μm). 1 or 2, more often 4 zoospores release from the cyst after excystment. Parasitoid of marine planktonic diatoms *Thalassiosira punctigera* (Castracane) found in Hasle from the North Sea.

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Parasites in algae mass culture

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Parasites are now known to be ubiquitous across biological systems and can play an important role in modulating algal populations. However, there is a lack of extensive information on their role in artificial ecosystems such as algal production ponds and photobioreactors. Parasites have been implicated in the demise of algal blooms. Because individual mass culture systems often tend to be unialgal and a select few algal species are in wide scale application, there is an increased potential for parasites to have a devastating effect on commercial scale monoculture. As commercial algal production continues to expand with a widening variety of applications, including biofuel, food and pharmaceuticals, the parasites associated with algae will become of greater interest and potential economic impact. A number of important algal parasites have been identified in algal mass culture systems in the last few years and this number is sure to grow as the number of commercial algae ventures increases. Here, we review the research that has identified and characterized parasites infecting mass cultivated algae, the techniques being proposed and or developed to control them, and the potential impact of parasites on the future of the algal biomass industry.

Keywords: algae mass culture, algae parasites, parasite detection, contamination control, algal biofuels

INTRODUCTION

Biological constraints on mass algae production in the form of grazers, pathogens and parasites are numerous (**Table 1**). Parasites have been recognized as important drivers of algae population regulation in nature. For example, populations of bloom-forming algae are often associated with parasites that, along with grazers and pathogenic bacteria, play an important role in the eventual demise of the bloom (e.g., Grami et al., 2011). Similar to high density algae blooms in nature, intensive algal production is likely to be associated with higher instances of disease outbreaks caused by pathogens and parasites, as seen with seaweed cultivation in Asia (Gachon et al., 2010). In fact, fungal contamination by chytrids has been recognized as one of the most serious hurdles for producing astaxanthin from the green algae *Haematococcus pluvialis* (Han et al., 2013). Undoubtedly, the wide array of known and yet to be characterized parasites associated with algae will pose a significant biological, and thus, economic challenge to the commercial cultivation of algae in industrial settings.

REVIEW OF SOME KNOWN ALGAE PARASITES

FUNGI

In freshwater environments, zoosporic fungi (Chytridomycota) and fungi-like organisms (including oomycetes, labyrinthulids, thraustochytrids and phagomyxids) are well known to parasitize microalgae. However, in marine systems the vast majority of important predators, pathogens and parasites have not been well characterized. In fact, it is estimated that only 0.6% of fungi studied are marine and these are only distantly related to known and cultured fungi (Richards et al., 2012). This suggests that there is an enormous amount of undiscovered marine microbial diversity. This is significant because the majority of algal species being utilized for biofuel production are marine in origin, posing severe monetary risk for the algal industry, which will have to contend with enemies it is not prepared for.

Members of the Chytridiomycota are extremely common fungal parasites in freshwater systems that prey on algae (**Table 1**). Their host ranges can be narrow (e.g., *Paraphysoderma sedebokerensis;* Hoffman et al., 2008; Gutman et al., 2009) or wide (e.g., *Rhizophydium algavorum*; Gromov et al., 1999) depending on the species. Chytrids produce motile dispersing life stages know as zoospores, are either saprotrophic or parasitic and are important contributors to aquatic food chains and carbon cycling (reviewed by Gleason et al., 2008). Despite high infection rates in natural algae populations, it is unclear how severe the effect of parasitic chytrids is (e.g., Kagami et al., 2011), however their impact may be amplified in commercial settings. For example, the chytrid *Phlyctidium scenedesmi* has been noted to cause severe production loss of *Scenedesmus* in open pond systems (Fott, 1967; Ilkov, 1975). Microbiome analyses and chitin staining recently detected several chytrids that co-occurred with loss of productivity of mixed green algae (family: *Scenedesmaceae*) growing in a prototype Offshore Membrane Enclosures for Growing Algae (OMEGA) system (Carney et al., 2014). These included the parasitic chytrid *Rhizophidium* sp. and a saprotrophic cytrid that was either *Powellomyces* or *Entophlyctis* sp. Unfortunately, the thick-walled cysts of chytrids can withstand many disinfection techniques (Fott, 1967), and once established may become a persistent problem for subsequent cultures.

Aphelids are a sister taxon of the chytrids in the Cryptomycota and are known as intracellular parasites that feed on microalgae (Karpov et al., 2013) (**Table 1**). A novel aphelid, *Amoeboaphelidium protococcarum*, was recently discovered and

Table 1 | Parasites reported for microalgae, including common group names and phyla, and the type of system the relationship was reported for.

Parasite				
Group/Taxonomy	**Species**	**Microalgal host**	**System**	**Citation**
Amoebae/*Endomyxa*	*Leptophyrs vorax*	*Closterium* sp.	Laboratory culture	Hess et al., 2012
Amoebae/*Endomyxa*	*Vampyrella* sp.	Various	Natural systems	Hess et al., 2012
Aphelid/*Cryptomycota*	*Amoeboaphelidium protococcarum*	*Scenedesmus* sp.	Open raceways for mass cultivation	Letcher et al., 2013
Aphelid/*Cryptomycota*	*Amoeboaphelidium protococcarum*	*Scenedesmus* sp.	Laboratory culture	Gromov and Mamkaeva, 1970
Chytrid/*Blastocladiomycota*	*Paraphysoderma sedebokerensis*	*Haematococcus pluvialis*	Laboratory culture	Hoffman et al., 2008; Gutman et al., 2009
Chytrid/*Chytridiomycota*	*Rhizophydium algavorum*	Various	Laboratory culture	Gromov et al., 1999
Chytrid/*Chytridiomycota*	*Chytriomyces* sp. and *Zygorhizidium* sp.	Various diatoms	Laboratory culture and natural systems	Canter and Jaworski, 1979; Beakes et al., 1988; Bruning, 1991; Grami et al., 2011; Kagami et al., 2011
Chytrid/*Chytridiomycota*	*Entoplyctis apiculata*	*Chlamydomonas* sp.	Natural system	Shin et al., 2001
Chytrid/*Chytridiomycota*	*Phlyctidium scenedesmi*	*Scenedesmus* sp.	Open raceways for mass cultivation	Fott, 1967; Ilkov, 1975
Chytrid/*Chytridiomycota*	*Rhizophidium* sp.	*Scenedesmaceae*	Closed photobioreactors for mass cultivation	Carney et al., 2014
Labyrinthulid/ *Labyrinthulomycota*	*Labyrinthula* Cienk.	*Cyanobacteria*	Natural system	Raghukumar, 1987
Oomycete/*Oomycota*	*Ectrogella* sp.	*Pseudo-nitzschia*	Natural system	Hanic et al., 2009
Oomycete/*Oomycota*	*Lagenisma coscinodisci*	*Coscinodiscus centralis*	Natural system	Gotelli, 1971
Sindinids/*Alveolata*	*Amoebophrya* sp.	*Dinoflagellate*	Natural system	Guillou et al., 2008; Chambouvet et al., 2011
Sindinids/*Alveolata*	*Amoebophrya ceratii*	*Dinoflagellate*	Natural system	Chambouvet et al., 2008

described infecting *Scenedesmus dimorphus* in commercial ponds in New Mexico, USA (Letcher et al., 2013). The proliferation of *A. protococcarum* in the ponds was correlated with a decrease in algae. This finding highlights that the need to identify new parasites will likely increase with the growth of the commercial algae industry.

Labyrinthulids are members of the fungal class *Labyrinthulomycetes*, commonly known as the slime molds, and include some important parasites of marine algae and plants (**Table 1**). Members of the genus Labyrinthula cause wasting disease in seagrasses and parasitize green algae and cyanobacteria (Raghukumar, 1986, 1987). These fungi have not yet been described in commercial systems, but closely related saprotrophic Thraustochytrids have been detected in *Nannochloropsis* sp. raceways using microbiome analysis (Carney et al., in preparation).

Oomycetes are a group of fungus-like organisms (technically in Kingdom Chromista) commonly referred to as water molds that include many known parasites of a wide variety of prey, including land plants and marine seaweeds and microalgae (Gachon et al., 2009; Hanic et al., 2009; Li et al., 2010) (**Table 1**). Oomycetes have caused losses to the seaweed industry that range from 10 to 60% annually for some countries (Gachon et al., 2010). Reported infection frequencies by the oomycetes *Ectrogella* and *Lagenisma* in natural populations of marine diatoms have ranged from <1 to 99% (Hanic et al., 2009, reviewed by Li et al., 2010). Like Labyrinthulids, Oomycetes have not yet been described in algae cultivation systems but their presence will likely be discovered as the commercial microalgae industry grows and molecular detection techniques are more frequently applied.

AMOEBEA

Vampyrellids are naked filose amoebae that perforate algae cell walls with spike-like pseudopodia in order to extract cellular content (Hess et al., 2012), hence the graphic name assigned to this group. Vampyrellids are common in freshwater and some are thought to be marine (**Table 1**). Although this group is not very well understood, Vampyrellids may become more notorious as the commercial algae industry grows.

OTHER PARASITES

The *Syndiniales* are alveolates, closely related to dinoflagellates, known to infect bloom-forming dinoflagellates in nature, exerting population regulation in only a few days by causing cell death without reproduction (reviewed by Gachon et al., 2010; Miller et al., 2012). *Amoebophrya* sp. were recently discovered to be able to survive in dormant cysts of their hosts for many months, causing immediate reinfection cycles as soon as the cysts emerged (Chambouvet et al., 2011), suggesting the infection by these parasites could be perpetuated very easily. *Amoebophrya ceratii* was recently discovered as the agent preventing seasonal algae blooms by an invasive dinoflagellate in an estuary in France (Chambouvet et al., 2008). However, the parasite was also found to infect every

other dinoflagellate species in the area, including many native species. The wide host range and recurrence of infection of the sindinids pose many challenges to commercial production of dinoflagellates.

DETECTION OF ALGAL PARASITES

MICROSCOPY AND STAINING

Calcofluor white is commonly used to visualize chytrids by staining the chitin in the cell walls of certain life stages (Kagami et al., 2004; Rasconi et al., 2009). Gerphagnon et al. (2013) proposed a double staining method to assess chytrid infection rates of cyanobacteria using Calcofluor white and SYTOX green, a nucleic acid stain. The authors used a combination of UV and blue light to show chytrid zoospores (green) inside sporangia (blue). However, for some algae Calcofluor white is problematic when cellulose is the primary cell wall component, such as for *Haematococcus pluvialis,* because cellulose can be stained as well as chitin and may obscure detection (Damiani et al., 2006). In addition, Calcofluor white cannot stain fungi lacking chitin. As an alternative, staining chytrid sporangia with nile red, even at very young stages, can be used as an early detection method for algae (Gutman et al., 2009). Congo red staining has been used to visualize oomycetes parasitizing seaweeds (Gachon et al., 2010) and may be useful for parasites of microalgae.

FLOW CYTOMETRY

Sieracki et al. (1998) have developed an automated flow cytometry and microscopy system known as FlowCAM (Fluid Imaging Technologies) for the enumeration and characterization of suspended particles, usually phytoplankton, in the 20–200 μM size range. The FlowCAM operates by flowing samples through a 3 × 3 mm glass chamber illuminated by a green laser and where relative fluorescence data, dimensions and a digital microscopic image of each particle are captured. Image analysis software, included with the instrument, carries out a pixel-based comparison correlation between captured images and a previously collected reference image set resulting in a percent similarity score. Images, that exceed a user determined similarity threshold, are considered to be a match for the target organism and can be displayed for visual confirmation by the user. In this manner FlowCAM systems can be used for semiautonomous identification and enumeration of target species. FlowCAM systems have been used in a variety of applications in marine and aquatic sciences including the detection and enumeration of the toxic dinoflagellate *Karenia brevis* in laboratory cultures, spiked natural phytoplankton assemblages and field samples from the Gulf of Mexico (Buskey and Hyatt, 2006). More recently FlowCAM analysis has been applied to the monitoring of algal mass culture systems for the early detection of algal predators (Day et al., 2012). A different flow-through microscopy system has been developed and demonstrated for the automated monitoring of cell count, size and morphology in microalgal culture (Havlik et al., 2013) To date there are no reports in the literature of the application of FlowCAM technology to the detection of parasites in algal mass culture but the technology may be applicable to visually distinctive species.

MOLECULAR–BASED DETECTION AND MONITORING

Modern molecular methods that have been developed for ecological studies can offer alternatives to optical based detection systems for the identification and detection of parasites in algal mass culture. Initial molecular identification can be carried out by Sanger sequencing of isolated DNA templates or by shotgun approaches based on next generation sequencing technologies. Once identified and characterized, systems that utilize amplification of or oligonucleotide hybridization to specific target regions can be employed for the detection of parasitic species. There are a variety of target regions that have been developed for either identification or detection; most of which utilize the ribosomal RNA (rRNA) encoding region. The three main targets in this region are the small subunit (SSU) rRNA large subunit (LSU) rRNA genes and the internal transcribed spacer (ITS).

In general, the SSU rRNA gene contains nine hypervariable regions (V1–9). However, unlike the prokaryotic SSU rRNA, the eukaryotic form lacks the V6 region so regions V4 and V9 are the most common individual hypervariable regions used for the phylogenetic analysis of eukaryotes (Amaral-Zettler et al., 2009; Stoeck et al., 2010; Pawlowski et al., 2011; Orsi et al., 2013). The V4 is the longest of the hypervarible regions, displays the highest degree of length variation and sequence heterogeneity (Nickrent and Sargent, 1991) and is generally sufficient for the genus level identification of an organism (see examples in Carney et al., 2014). The shorter V9 region is sometimes used in combination with V4 but, since it lies at the extreme 3′ terminus of the SSU rRNA gene, it is often not included in less than full-length amplicons used for sequencing. Thus, data is often missing for the V9 region in nucleotide sequence database entries. Two variable regions (D1 and D2) in the LSU rRNA gene are also used for phylogenetic analysis (Ludwig and Schleifer, 1994; Sonnenberg et al., 2007; Putignani et al., 2008). A number of tools have been developed to assist in rRNA based phylogenetic analysis. Perhaps the most heavily utilized of these is the SILVA database, (http://www.arb-silva.de/), a curated and downloadable repository of SSU and LSU rRNA gene sequences (Quast et al., 2013). The SILVA website also includes webtools for the *in silico* testing of primer and probe sequences for specificity. In adddition, probeCheck, http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home (Loy et al., 2007) can also be used in a similar fashion to aid in the design of SSU and LSU rRNA probes.

For finer levels of phylogenetic discrimination, the ITS region is commonly utilized. This region consists of two hypervariable spacers, ITS1and ITS2, 5′, and 3′ of the gene encoding the 5.8 s ribosomal subunit. Currently there are three online databases of ITS sequences: UNITE (http://unite.ut.ee/) contains primarily fungal ITS regions (Abarenkov et al., 2010), the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/; Koetschan et al., 2012), and ITSoneDB, (http://itsonedb.ba.itb.cnr.it/) focuses on the ITS1 region.

A full-length amplicon including both ITS1 and ITS2 regions and the 5.8 s subunit is approximately 650 bp in length, which is beyond the current read length limits of many of the next generation sequencers. Consequently, individual ITS regions have been analyzed by next generation sequencing (Lindner et al., 2013). Reports indicate that community analyses based on ITS1 vs. ITS2

yield different taxonomic compositions from each other as well as from those based on the full length ITS region.

Because of its potential for high sensitivity, quantitative PCR (qPCR) is a powerful and widely employed detection method (for review of qPRC detection of waterborne agents see Botes et al., 2013). Careful sample preparation methods including primer design and validation and selection and testing of reagents are required in order to limit background contamination and achieve the highest levels of sensitivity. qPCR reactions can be multiplexed for the simultaneous detection of multiple species within a single reaction. qPCR has been used in commercial algae production to quantify changing densities of algae and their parasites in mass-culture, for example, *Amoeboaphelidium protococcarum* (Letcher et al., 2013) and chytrids (Shurin et al., 2013). qPCR could be a very important detection tool when the parasite is known. Alternative detection methods include hybridization-based systems, such as the phylochips, which utilize arrayed oligonucleotide probes (Loy et al., 2002; Metfies et al., 2007). Although, without an integrated amplification step, these methods are not as sensitive as qPCR; they are however designed to be highly multiplexed for the simultaneous detection of a diversity of species and thus could be both cost and time effective, often required in commercial settings.

SOLUTIONS TO PARASITE CONTAMINATION

SALVAGE HARVEST

Perhaps the most obvious, least technologically demanding and least satisfying response to parasite contamination is salvage harvest. This is, of course, to simply harvest the algal biomass upon detection of a parasitic species and prior to serious loss of biomass. Successful salvage harvest is dependent on both the early detection and quantification of the contaminating parasite and the operator experience necessary to determine optimal harvest time by balancing the maximizing of biomass yield against the potential for catastrophic loss. Although this method reduces the impact of an infection, it still results in removal of a mass culture system from active production. The system must be disinfected prior to return to production or used to produce an alternative, non-susceptible species. Disinfection of unlined, open pond systems can be challenging and techniques may be limited to drying and exposure to solar radiation.

CHEMICAL AGENTS

Natural defenses of algae to parasite infections include abscisic acid (ABA) production during some life cycle stages (Pouneva, 2006). ABA may also have protective effects when applied exogenously. An anti-fungal protein isolated from a marine bacterium has been used to protect commercially grown red seaweed from the oomycete causing red rot disease (Woo and Kamei, 2003). The addition of copper sulfate to growth media has been reported to both stimulate algae productivity and serve as a fungicide against chytrids (Fott, 1967). An unspecified chemical fungicide was successful at controlling chytrid densities in commercial ponds of *Scenedesmus* sp. (Shurin et al., 2013). The surfactant Triton-N was proposed for treating algae inoculum before scale-up when it is still a small volume in order to reduce chytrid productivity (Benderliev et al., 1993). Several commercially available disinfectants were found to control the spread of the chytrid infecting amphibians worldwide, *Batrachochytrium dendrobatidis* (Webb et al., 2012) and may have application to algal mass culture.

Ozone treatment has been employed for the destruction of invasive species in ballast water but the application of the method to algal parasites has not been reported (Tsolaki and Diamadopoulos, 2010). Germicidal ultra violet radiation subtype C (UVC; 280–100 nm wavelength) has also been proposed for the control of invasive species but has not been demonstrated for use against algal parasites (Liebich et al., 2012). Successful employment of UVC in contaminated mass algal cultures would depend on greater resistance to irradiation of the alga vs. the parasite. This appears to be possible because of the greater level of pigmentation in microalgae vs. many parasite species. Alternatively UVC could be utilized to eliminate sources of contamination in liquid nutrient stocks or source water for cultivation.

PHYSICAL METHODS

Physical disruption (sonication) has been studied for the destruction of invasive and deleterious organisms in ballast water (Holm et al., 2008). Dose is dependent on cell size with larger organisms requiring less energy input for disruption than those that are smaller. This makes sonication particularly attractive for the control of predators but potentially less so for the parasites, which may not display as great a size differential. Physical removal methods such as screening have also been demonstrated for the removal of large biovolume predators such as rotifers but, again, this dependence on size differential may limit its utility in treating parasite infections.

BIOLOGICAL CONTROL

Selective breeding/modification

The utilization of selective breeding, or other methods for the genetic manipulation of microalgae, to develop resistance to parasite infection in algal mass culture has yet to be reported. Microalgae have several characteristics that could potentially lend themselves to such an approach (for review see Larkum et al., 2012). Generally speaking, generation times are relatively short and UV or chemical mutagenesis methods have been developed in a number of species (Huesemann et al., 2009). In a handful of strains, including *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Thalassiosira psuedonana*, *Nanochloropsis salina*, and *Dunaleiella* sp., transformation methods have been developed enabling the genetic engineering of these species (for review see Qin et al., 2012). The success of any genetic manipulation technique is dependent on the ability to select for resistance; such selection would require that the target parasite species is maintained in culture. Other limitations to genetic modification approaches include the potential for tradeoffs; a mutant microalgal strain that demonstrates good parasite resistance may not perform well in other aspects. The breadth of resistance could be quite narrow with resistance to one parasite species extending only to closely related species. Finally, because of selective pressure, there is the potential for the parasite to rapidly evolve to overcome the host algae resistance.

Biological agents

Bio-control is widely used in agriculture and on public lands to manage unwanted pests ranging from invasive plants to mammals. Bio-control of plant pathogens has been gaining momentum (36% of existing bio-control agents were developed only in the last 5 years) and is often preferred over chemical means of control. Residues from chemical control methods may hinder downstream product processing, regulation of these chemicals has become increasingly restrictive, and target pests are likely to develop chemical resistance (Fravel, 2005). Alternatively, organisms that prey on and parasitize microalgae have their own set of predators and parasites that may be used to control them in commercial settings.

Zooplankton may be used as an effective control of algae parasites as they prey on fungal spores, particularly chytrid zoospores (Kagami et al., 2004), which have been recognized as important resources in natural food chains (Gleason et al., 2008). Due to its efficient grazing of chytrid zoospores, *Daphnia* has been tested in mesocosms as a potential biocontrol agent of *Batrachochytrium dendrobatidis* (Hamilton et al., 2012). However, in this example, Daphnia also served as prey for developing tadpoles (Hamilton et al., 2012). Care must be taken in applying zooplankton to control fungus in algae cultures as many of these also rapidly ingest algae and can have equally, if not more, damaging effects on algae biomass than chytrid infections do.

Hyperparasites are organisms that parasitize other parasites, although these have not been studied very thoroughly. Work is needed to describe algae-parasite-hyperparasite relationships that may be common to commercial algae production ponds and that show promise as a solution paradigm to fungal infections of algae and from which future bio-control tools can be developed for additional algal predators. Evidence suggests hyperparasite infections do not kill their fungal host but instead reduce their reproductive success by efficiently co-opting the cytoplasm of infected cells and thereby indirectly reducing infection rates of algae (Gleason et al., 2012). Examples of such relationships are common in freshwater environments and a marine example has been described (Kagami et al., 2007; Gleason et al., 2012). In addition, metagenomic evidence suggests hyperparasites are common in marine communities (James and Berbee, 2011). The host range of hyperparasites has been determined to be narrow with each species of hyperparasite infecting only closely related species. Some hyperparasites produce resting spores that can withstand desiccation and may potentially be used to inoculate cultures of infected algae in order to control the fungal parasite. In fact, fungal species that have resting spores as part of their life cycles are noted as being easy to formulate as bio-control agents because they are easier to ship and have lower risk of contamination by bacteria and other fungus (Fravel, 2005). As our interest, and perhaps future dependence, on biofuels grows, the complex algal pond relationships will need to be understood and controlled in order to attain sufficient algal productivity.

Allelopathy is the production of one or more biochemicals by an organism that affects, either positively or negatively, the survival, growth or reproduction of another species. Negative allelopathy has been proposed as a potential method of controlling deleterious species in algal mass culture systems (Mendes and Vermelho, 2013). However, alleopathic relationships between microalgae and parasites have not been reported and thus allelopathy is only a hypothetical mechanism for the control of parasites in algal mass culture.

CONCLUSIONS

Here we have reviewed some known parasites of microalgae, as well as some taxonomic groups that will likely become better known (i.e., infamous) as commercial production of microalgae increases worldwide. Members of the fungi, including chytrids and oomycetes, appear to be the most common and potentially, least controllable, group of parasites. Early detection of parasites is essential to ensure the efficacy of possible treatments of the infection. Traditional detection methods such as microscopy and staining can be used to visualize algal parasites, however this technique may be too labor intensive to perform on a routine basis for most commercial operations. For routine detection, more automated systems would be ideal (i.e., flow cytometry). If the budget allows, molecular-based techniques are the most informative and sensitive for the purposes of identifying which parasites may be present using Sanger, shotgun or next generation sequencing and then monitoring for these specifically using qPCR or phylochip technology.

The economic feasibility of the various parasite detection and control methods is largely driven by two factors; the volume of the algal mass culture system and value of the final product or products. Algal biofuels is a prime example of a low value product that must be produced at large scale. To compete with gasoline at $0.53US per liter it will be necessary to produce dry algal biomass at $0.14US per kilogram (Sun et al., 2011). Given these limitations, it is widely, but not universally, held that large-scale open ponds may be the only economically feasible means of production. Because of this combination of the large scale of cultivation, the relatively low productivities that can be achieved in open systems (as opposed to closed PBRs), and the low value of the final product, the options for parasite control in biofuels applications are economically constrained. In addition, the likelihood of infection is the greatest in open systems. In such an application, intervention strategies must be targeted and inexpensive. Conversely, higher value products such as nutraceuticals, may accommodate a larger range of parasite control strategies while remaining economically viable. These operations, which tend to feature lower volume cultivation units, can take advantage of broadly applied prophylactic methods such as filtration, UV and chemical pretreatment of the source water.

In terms of low cost countermeasures, salvage harvest may, of course, be the least expensive but least satisfactory method of intervention. The obvious limitations of this practice continue to fuel the drive to seek alternative crop protection strategies. In the near-term, intervention with various biocidal chemicals is likely to be the most effective alternative. This is largely driven by the fact that several such chemicals already exist and are utilized in terrestrial agriculture. However, if the final products or co-products are intended for human or animal consumption, this may limit the application of chemical countermeasures. In addition, it may be economically infeasible to routinely treat all

cultivation units and such extensive use of chemicals could result in the development of resistant parasite strains.

It is likely that the best long-term strategy for control of parasites in production facilities will take the form of an integrated pest management strategy. Such a strategy would include the cultivation of resistant strains, the limited use of chemical agents, the development of biological control systems and crop rotation to limit the accumulation of parasites or the development of resistance to countermeasures. By combining different management strategies, shortcomings of any one strategy may be overcome and reliance on chemicals can be reduced (reviewed by Chandler et al., 2011). Resistant algal strains could be developed either by classical mutagenesis and selection strategies or by genetic engineering techniques. The former would not be considered a genetically modified organism (GMO) for regulatory purposes.

Routine monitoring and early detection of pest species is a clear requirement for large-scale cultivation. Knowledge gained from long-term operations will allow for the identification of common pest species and the environmental conditions in which they are most prevalent. To some degree this gives the operators predictive capability. In that manner only the cultivation units that require it are treated. Daily microscopic analysis is a standard practice at many production facilities. However, this process is labor intensive and requires a certain degree of expertise. Alternative parasite detection method based molecular assays such as PCR will likely find application in the production of high value products but do require significant capital outlay and may be too expensive for large-scale operations. The same may be said of image recognition systems with the additional caveat that they may be unable to identify morphologically indistinct species. Clearly there is an unmet need in the nascent algal production industry for a low cost user-friendly "dipstick" assay for the major parasite species. In addition, more work is needed in developing treatment protocols that target specific host-parasite relationships, as both the parasite and some algal hosts may be affected negatively by the treatment.

Despite a paucity of publically available data on the economic impact of parasitism on the nascent algae biomass industry, the consensus is that biocontaminants, in general, constitute an economic barrier to commercialization (ANL et al., 2012; Gao et al., 2012). Some insight into the potential magnitude of the financial impact may be gained from the *Porphyra* (nori) industry in asia which loses 10%, on average, of its annual production to parasitism by oomycetes, with losses up to 64% in certain regions during some years (reviewed by Gachon et al., 2010). Commercial algal mass culture operations would clearly benefit from a more complete understanding of algal parasites including regional, environmental and seasonal variation in occurrence of parasite infestations and a characterization of the susceptibility of common production strains to different parasites. Such an understanding would require a systematic approach to the analysis and characterization of pond infections and a certain amount of data sharing among pond operators. At minimum, a shared database of molecular probes and PCR primer sequences for detection of parasites would be beneficial. Perhaps as the algal mass culture industry becomes more economically feasible and therefore more important there will be greater impetus to take such an approach to the problem.

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Ecological functions of zoosporic hyperparasites

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Zoosporic parasites have received increased attention during the last years, but it is still largely unnoted that these parasites can themselves be infected by hyperparasites. Some members of the Chytridiomycota, Blastocladiomycota, Cryptomycota, Hyphochytriomycota, Labyrinthulomycota, Oomycota, and Phytomyxea are hyperparasites of zoosporic hosts. Because of sometimes complex tripartite interactions between hyperparasite, their parasite-host, and the primary host, hyperparasites can be difficult to detect and monitor. Some of these hyperparasites use similar mechanisms as their parasite-hosts to find and infect their target and to access food resources. The life cycle of zoosporic hyperparasites is usually shorter than the life cycle of their hosts, so hyperparasites may accelerate the turnaround times of nutrients within the ecosystem. Hyperparasites may increase the complexity of food webs and play significant roles in regulating population sizes and population dynamics of their hosts. We suggest that hyperparasites lengthen food chains but can also play a role in conducting or suppressing diseases of animals, plants, or algae. Hyperparasites can significantly impact ecosystems in various ways, therefore it is important to increase our understanding about these cryptic and diverse organisms.

Keywords: hyperparasites, ecology, food web, parasite, zoospores, eDNA

INTRODUCTION

"So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed ad infinitum."
Jonathan Swift, On Poetry: a rhapsody (1733)

Parasites belonging to all taxonomic groups have gained increasing attention in ecological research during recent years. It is widely recognised that the number of species of parasites are more numerous than organisms with a non-parasitic lifestyle (Lafferty et al., 2008). Also it is widely accepted that many parasites can themselves be hosts for other parasites. Such parasites of parasites are usually called "hyperparasites"; a term which is used without any reference to the phylogeny of the host or the parasite or whether the relationship is obligately or facultatively parasitic. Novel methodological tools and an increasing interest in parasites and their ecology have led to more targeted sampling approaches. This has shown that especially microbial parasites which have until now been rarely detected are abundant and diverse (Lefèvre et al., 2008; Jones et al., 2011; Hartikainen et al., 2014). It is very difficult—or in many cases impossible—to isolate and identify them because of their generic morphology, and because such parasites are often restricted to only a few host cells which makes them difficult to detect even with state of the art molecular methods. Hence, it is no surprise that microbial hyperparasites are not well understood. Some species of hyperparasites are endoparasites and difficult to see in the light microscope without special staining methods. Although zoosporic parasites of primary producers have been the focus of recent studies (Powell, 1993; Ibelings et al., 2004; Kagami et al., 2007; Marano et al., 2011; Neuhauser et al., 2011a), our knowledge about zoosporic hyperparasites and their microbial hosts remains anecdotal. In this article we focus on zoosporic hyperparasites with zoosporic hosts, their abundance and relationships between parasites and their hosts and their possible roles in ecological processes.

In two of the early works focusing on microbial hyperparasites, Karling (1942a,b) documented and discussed examples of hyperparasitism among zoosporic true fungi (**Table 1**). Although his study focused primarily on hyperparasites among the zoosporic true fungi, Karling was aware of hyperparasites among other microbial groups such as stramenopiles or plasmodiophorids (**Table 2**). Sparrow's monograph about aquatic phycomycetes contains still the most comprehensive references to zoosporic hyperparasites (Sparrow, 1960). Although hyperparasitism among true fungi has been the focus of numerous research projects, for instance in the form of biological control of plant diseases (e.g., Vinale et al., 2008), hyperparasitism involving heterotrophic stramenopiles and zoosporic true fungi has been rare (Boosalis, 1964; Barnett and Binder, 1973; Adams, 1990). Zoosporic hyperparasites have been described

Table 1 | Selected hyperparasitic Opistokonts (Chytridiomycota, Cryptomycota, Blastocladiomycota).

Hyperparasite	Trophic mode	Parasite (=Host of hyperparasite)	Host (=Host of parasite)	References
Cryptomycota		**Chytridiomycota**		
Rozella marina	Biotroph	*Chytridium polysiphoniae*	Parasite, red algae	Sparrow, 1960; Held, 1981
Rozella parva	Biotroph	*Zygorhizidium affluens*		Canter, 1965; Beakes et al., 1988
Rozella rhizophlyctii	Biotroph	*Rhizophlyctis rosea*	Facultative parasite	Karling, 1960; Held, 1981
	Biotroph	*Rhizophydium globosum*	Parasite, Diatoms, algae	Sparrow, 1960; Held, 1981
Rozella polyphagi	Biotroph	*Polyphagus laevis*	Parasite, *Euglena*	Sparrow, 1960; Held, 1981
	Biotroph	*Polyphagus euglenae*	Parasite, *Euglena*	Powell, 1984
Rozella endochytrium	Biotroph	*Endochytrium operculatum*	Facultative parasite, algae	Sparrow, 1960; Held, 1981
Rozella cladochytrii	Biotroph	*Cladochytrium replicatum*	Facultative parasite, green algae	Sparrow, 1960; Held, 1981
Cryptomycota		**Blastocladiomycota**		
Rozella allomycis	Biotroph	*Allomyces arbuscula*	Facultative parasite, insect cadaver	Held, 1981
	Biotroph	*Allomyces macrogynus*		Held, 1974
Cryptomycota		**Oomycota**		
Rozella rhipidii-spinosi	Biotroph	*Araiospora spinosa*	Facultative parasite	Sparrow, 1960; Held, 1981
Rozella apodiae-brachynematis	Biotroph	*Apodachlya brachynema*	Facultative parasite	Sparrow, 1960; Held, 1981
Rozella achlyae	Biotroph	*Achlya flagellata*	Facultative parasite	Sparrow, 1960; Held, 1981
		Dictyuchus anomalus	Parasite, fish	
Rozella cuculus	Biotroph	*Pythium intermedium*	Parasite, plant	Sparrow, 1960; Held, 1981
		P. monospermum	Parasite, nematode	Held, 1981
Rozella laevis	Biotroph	*Pythium gracile*	Parasite, green algae	Sparrow, 1960; Held, 1981
Rozella barrettii	Biotroph	*Phytophthora cactorum*	Parasite, plant	Sparrow, 1960; Held, 1981
Rozella pseudomorpha	Biotroph	*Lagenidium rabenhorstii*	Parasite, green algae	Sparrow, 1960; Held, 1981
Chytridiomycota		**Chytridiomycota**		
Dictyomorpha dioica	Biotroph	*Achlya flagellata*		Mullins and Barksdale, 1965
Chytridium parasiticum	Biotroph	*Septosperma rhizophydii*	Parasite, chytrid	Karling, 1960
Rhizophydium parasiticum		*Rhizophlyctis rosea*	Facultative parasite, chitin	Karling, 1960; Sparrow, 1960
		Chytridiomyces verrucocsa		
Rhizophydium carpophilum		*Synchytrium fulgens*	Parasite, plant	Karling, 1960
		S. macrosporum	Parasite, plant	
		S. linariae	Parasite, plant	
Phlyctochytrium synchytrii		*Synchytrium endobioticum*	Parasite, plant	Karling, 1942a
Septosperma rhizophydii		*Rhizophydium macrosporum*	Facultative parasite	Karling, 1960
Septosperma anomala		*Phlyctidium bumelleriae*	Parasite, Xanthophyceae	Karling, 1960
Chytridiomycota		**Oomycota**		
Rhizophydium pythii	Biotroph	*Pythium monospermum*	Parasite, nematode	Sparrow, 1960
Rhizidiomyces japonicus		*Phytophthora megasperma*	Parasite, plant	Sneh et al., 1977
		Phytophthora erythroseptica	Parasite, plant	Wynn and Epton, 1979
Canteriomyces stigeoclonii		*Phytophthora megasperma*	Parasite, plant	Sneh et al., 1977
Blastocladiomycota		**Oomycota**		
Catenaria anguillulae	Facultative	*Phytophthora cinnamomii*	Parasite, plant	Daft and Tsao, 1984
		Phytophthora parasitica	Parasite, plant	

Hyperparasites and hosts are sorted by taxon. Higher ranks are given in bold.

in the fungal groups Chytridiomycota, Blastocladiomycota, and Cryptomycota (Opisthokonts, for examples see **Table 1**). Within the heterokonts the groups Hyphochytriomycota, Oomycota, Labyrinthulomycota, and Phytomyxea contain hyperparasitic species (**Table 2**). These groups belong to various supergroups in the tree of life (Baldauf, 2003; Adl et al., 2012), but these microorganisms interact together in the same ecosystems. Because of their morphological similarity and their similarity in size they can have ecologically similar functions and are in food web studies often treated as "trophic species" (Powell, 1993; Marano et al., 2011). Many of the known hosts belong to common genera which are frequently observed in many soil and fresh

Table 2 | Selected hyperparasitic Heterokonts (Oomycota, Hyphochytridiomycota, Phytomyxea).

Hyperparasite	Trophic mode	Parasite (=Host of hyperparasite)	Host (=Host of parasite)	References
Oomycota		**Oomycota**		
Olpidiopsis incrassata		*Saprolegnia ferax*	Parasite, fish	Slifkin, 1961
Olpidiopsis karlingiae		*Rhizophlyctis rosea*	Facultative Parasite	Karling, 1960
Pythiella vernalis		*Pythium aphanidermatum*	Parasite, plant	Pires-Zottarelli et al., 2009
		Pythium gracile	Parasite, green algae	Blackwell, 2010
Pythiella pythii		*Pythium dictyosporum*	Parasite, green algae	Blackwell, 2010
Pythium proliferum		*Rhizophlyctis rosea*	Facultative Parasite	Karling, 1960
Pythium monospermum		*Phytophthora megasperma*	Parasite, plant	Humble and Lockwood, 1981
Pythium oligandrum		*Pythium irregulare*	Parasite, plant	Ribeiro and Butler, 1995; Benhamou et al., 1999
		Pythium mamillatum	Parasite, plant	
		Pythium paroecandrum	Parasite, plant	
		Pythium aphanidermatum	Parasite, plant	
		Pythium sylvaticum	Parasite, plant	
		Pythium ultimum	Parasite, plant	
Hyphochytridiomycota		**Oomycota**		
Hyphochytrium catenoides	Facultative	*Pythium myriostylum*	Parasite, plant	Ayers and Lumsden, 1977
		Aphanomyces euteiches	Parasite, plant	Ayers and Lumsden, 1977; Sneh et al., 1977
		Phytophthora erythroseptica	Parasite, plant	Wynn and Epton, 1979
		Phytophthora megasperma	Parasite, plant	Humble and Lockwood, 1981
Phytomyxea		**Oomycota**		
Sorodiscus cokeri	Biotroph	*Pythium proliferum*	Facultative Parasite	Goldie-Smith, 1951
		Pythium graminicolum	Facultative Parasite, moss	Goldie-Smith, 1951
		Pythium catenulatum	Facultative Parasite, plant	Goldie-Smith, 1951
		Pythium elongatum	Facultative Parasite	Goldie-Smith, 1951
		Pythium irregulare	Parasite, plant	Goldie-Smith, 1951
		Pythium undulatum	Parasite, plant	Goldie-Smith, 1951
Woronina polycystis	Biotroph	*Saprolegnia ferax*	Parasite, fish	Goldie-Smith, 1954
Woronina pythii	Biotroph	*Pythium proliferum*	Facultative Parasite	Goldie-Smith, 1956a
		Pythium aphanidermatum	Parasite, plant	Goldie-Smith, 1956a
		Pythium debaryanum	Parasite, plant	Goldie-Smith, 1956a
		Pythium irregulare	Parasite, plant	Goldie-Smith, 1956a
		Pythium monospermum	Parasite, nematode	Goldie-Smith, 1956a
		Pythium pulchrum		Goldie-Smith, 1956a
		Pythium ultimum	Parasite, plant	Goldie-Smith, 1956a

Hyperparasites and hosts are sorted by taxon. Higher ranks are given in bold.

water ecosystems using both baiting procedures and molecular analysis of environmental samples (Sparrow, 1960; Powell, 1993; Barr, 2001; Dick, 2001; Lozupone and Klein, 2002; Shearer et al., 2007; Lefèvre et al., 2008; Marano et al., 2011). It is very likely that zoosporic hyperparasites are as abundant on "rarer" hosts. This is of ecological importance because zoosporic true fungi and heterotrophic stramenopiles can be among the predominant groups in some ecosystems (Lefèvre et al., 2008; Freeman et al., 2009; Marano et al., 2011). Because of the large number of species of zoosporic parasites, hyperparasites, and their associated hosts, it is likely that there are many additional taxa that await discovery.

ZOOSPORES

Zoospores are a shared morphological feature of the hosts and hyperparasites discussed here. Zoospores are motile propagules which permit rapid dispersal. Zoospores can sense environmental gradients which they use to identify and find potential hosts (Tyler, 2002). There are different types of zoospores (Lange and Olson, 1983), which have distinguishing features, allowing observers to determine and categorize the organisms. The most important feature is the type of flagellation. Zoospores can generally be grouped into (1) uniflagellate with posteriorly directed whiplash flagellum, (2) uniflagellate with an anteriorly directed tinsel flagellum, (3) biflagellate, heterokont, with one posteriorly

directed whiplash flagellum and one anteriorly directed tinsel flagellum and (4) biflagellate, isokont, two whiplash flagellae, often of different lengths, with the shorter one anteriorly directed and the longer one posteriorly directed.

Despite their relatively simple morphology many zoosporic hyperparasites form functionally and developmentally distinct types of zoospores during their life cycle (Sparrow, 1960). A variety of names are used for different types of zoospores in different taxonomic groups, but generally one type of zoospore is formed in zoosporangia following mitosis and can be either haploid or diploid, while another type of zoospore is formed by meiosis and is haploid (Lange and Olson, 1983). The different types of zoospores can serve different functions during the parasite life cycle—such as rapid propagation and dispersal or primary infection and population establishment after periods of hibernation (e.g., Neuhauser et al., 2011b). Despite variable modes of formation and complex parasite life cycles which can result in periods where one type of zoospore is predominantly formed, the main unifying feature of all types of zoospores is that they are small, single-celled, motile propagules. Within food webs zoospores provide a rapid energy source for a variety of organisms at higher trophic levels (Gleason et al., 2011), so it is not surprising that zoospores are often treated as trophic species.

MECHANISMS USED BY HYPERPARASITES TO ACCESS FOOD RESOURCES

Zoosporic hyperparasites use a large variety of mechanisms to attack their hosts. Hyperparasites can grow epibiotically on the surface of their host only entering the host cell with specialized structures such as chytrid rhizoids (**Figures 1A,C**). Hyperparasites also grow endobiotically this means completely submerged in their hosts (**Figures 1B,D**). The parasite hosts of hyperparasites can be ectoparasites (**Figures 1A,B**) growing epibiotically on the primary host or endoparasites (**Figures 1C,D**) growing endobiotically inside the primary host. Hyperparasites which are infecting ectoparasites only have to overcome the defense mechanisms of their host, and often use infection strategies that are very similar to those of zoosporic parasites (Sparrow, 1960; Marano et al., 2012). On the other hand, hyperparasites which are parasites of endoparasites may have to overcome two barriers of defense—they have to enter the parasite-host and their host to get access to food resources. Most of the described zoosporic hyperparasites are parasites of ectoparasites (e.g., most *Rozella* species, *Wornina* spp.). We hypothesize that ectoparasites are easier accessible for hyperparasites with only one line of defense to break. We also hypothesize that our knowledge about zoosporic hyperparasites of endoparasites is biased by the fact that zoosporic endoparasites are a poorly studied group themselves. Therefore, most of the examples discussed here are from zoosporic hyperparasites of parasites which are not completely submerged inside their host or from endoparasitic hyperparasites of epibiotic hosts (**Figures 1A–C**).

An example of an epibiotic infection (**Figure 1A**) is the parasitic relationship between the two chytrids *Chytriomyces verrucosus* and *Rhizophlyctis rosea* (Karling, 1960). The chemotactic zoospores of *R. rosea* are attracted to the host cell where they encyst. The zoospore then germinates and a germ tube penetrates

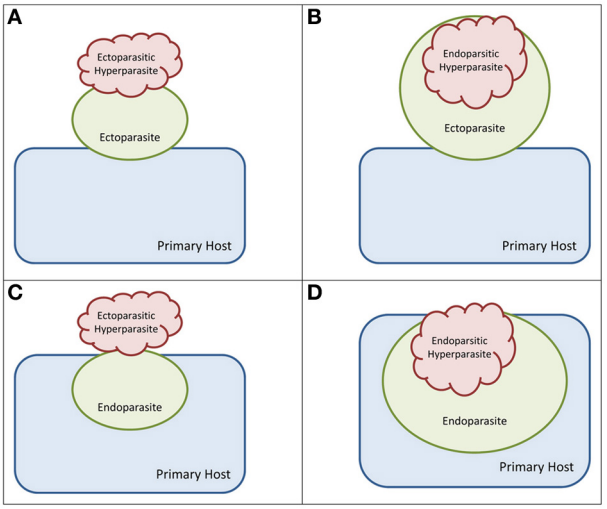

FIGURE 1 | Types of hyperparasitism. Blue—primary host, green—parasite, red—hyperparasite. **(A)** epibiotic hyperparasite of ectoparasite. This type can be found for example in the interaction of the hyperparasite *Rhizophydium parasiticum* (Chytridiomycota), its and its (facultative) parasites host *Rhizophlyctis rosea*. **(B)** Endobiotic hyperparasite of ectoparasite host. This is the most commonly described mode of hyperparasitsm seen in many *Rozella* species (Cryptomycota) or *Woronina* spp. (Phytomyxea). **(C)** Epibiotic hyperparasite of endoparasite host. E.g., *Rhizophyidum carpophilum* (Chytridiomycota) on *Olpidiopsis* sp. (oomycetes) and *Synchytrium* sp. (chytrid). **(D)** Endobiotic hyperparasite of endoparasite host. E.g., the hyperparasitic chytrid *Phlyctochytrium synchytrii* in the plant pathogen *Synchytrium endobioticum*.

the host zoosporangium. Inside the host, an endobiotic rhizoidal system develops supplying the epibiotic zoosporangium (having since formed from the body of the zoospore) with nutrients. Epibiotic parasites can also be found in the stramenopiles (Sneh et al., 1977): zoospores of the hyphochytriomycete *Rhizidomyces japonicus* attach to the surface of oospores of *Phytophthora megasperma* (Oomycetes) where thalli grow externally around the oospore and produce zoosporangia. The oomycete *Pontisma lagenidioides* which is a parasite of the green alga *Chaetomorpha media* can be infected by *Labyrinthula* sp. (Raghukumar, 1987).

Endobiotic parasites grow entirely submerged within their host. An example is *Rozella allomycis* (Rozellida/Cryptomycota) and its host *Allomyces arbuscula* (Blastocladiomycota) (Held, 1973, 1974). In this case, the infection process is relatively well studied and is described in more detail here to exemplify the infection process of most known endobiotic zoosporic hyperparasites. Substances produced by the host attract the chemotactic zoospores of the parasite toward the host. Once the zoospore attaches to the surface of the host cell it forms a so-called cyst, which produces a germ tube. The germ tube then grows into the host cell through the cell wall while the protoplast of *Rozella* is pushed into the host cell by fluid pressure produced from a vacuole in the cyst. Subsequently the parasite grows inside the host cell. In the case of *Rozella allomycis* the host cell is then transformed into the parasite sporangium. Other known endobiotic parasites are *Rozella polyphagi* (Rozellida/Cryptomycota), which parasitizes the chytrid parasite *Polyphagus euglenae*

(Powell, 1984) and the endobiotic parasite *Catenaria allomycis* (Blastocladiomycota), which infects *Allomyces javanicus* (Sykes and Porter, 1980; Powell, 1982). *Catenaria anguillulae*, a member of the Blastocladiomycota, is an endobiotic parasite of the plant pathogenic oomycetes *Phytophthora cinnamomi* and *P. parasitica* (Daft and Tsao, 1984), while *Hyphochytrium catenoides* (Hyphochytriomycota) colonizes oospores of *Pythium myriostylum* (Ayers and Lumsden, 1977). Another parasite of *Pythium* spp. is *Woronina pythii* (Phytomyxea), which infects both vegetative hyphae and reproductive structures of *Pythium* (Dylewski and Miller, 1983).

Interactions are slightly different between hyphal forming zoosporic organisms, such as oomycetes. Here interactions between hyphae can be observed, and these interactions are different from the endo- and epibiotic parasitic interactions discussed above. Two distinct mechanisms appear to be involved in interactions between this parasite and its hosts: (1) hyperparasitism; mediated by hyphal interactions, and (2) antibiosis; causing metabolic and developmental changes prior to contact between hyphae of the parasite and host (Adams, 1990; Benhamou et al., 1999). An example of direct interactions between the organisms is the interaction between hyphae of the well-known hyperparasite *Pythium oligandrum* (Oomycota) and hyphae of its oomycete hosts (e.g., *P. ultimum, P. aphanidermatum, Phytophthora megasperma*) (Benhamou et al., 1999). Hyphae of the parasite can adhere to the surface of the host sometimes coiling around the host hyphae. Penetration of the host cells by infection pegs may follow, leading to digestion of the host cytoplasm. When the interaction is initiated by antibiosis (without contact with the host) the parasite can release soluble substances which cause biochemical changes within the host cells. Then the parasite can release extracellular enzymes, which digest the host cells.

BIODIVERSITY AND HOST RANGE OF HYPERPARASITES

DNA sequences assigned to putative parasite and hyperparasite taxa of zoosporic fungi are widespread (e.g., Lara et al., 2010; Jones et al., 2011; Lara and Belbahri, 2011; Nagano and Nagahama, 2012). But molecular methods are often biased by the selection of primers and sampling methods (Hartikainen et al., 2014; Neuhauser et al., 2014) and the assignment of environmental DNA sequences to described species is only as good as the available reference datasets. Data on zoosporic microorganisms are sparse, and many of the "unknown" sequences are probably from common species which to date have no reference record in public data bases (e.g., Nagy et al., 2011; Karpov et al., 2013). Reliable reference sequences of many zoosporic hyperparasites are generally rare. One reason is that many of the known zoosporic hyperparasites are biotrophic parasites which cannot be grown without their hosts. The hosts themselves are often biotrophic parasites as well, making it very hard to isolate, identify and sequence the hyperparasites. Therefore, targeted studies to detect and characterize hyperparasites and their hosts are needed. Such targeted approaches could include baiting experiments combined with microscopic observation or DNA and RNA based screenings of various environments. Despite being very time consuming baiting and isolation experiments are highly valuable because they will allow to understand how hyperparasites interact with their hosts, to describe their life cycle, and to analyze interactions with their hosts. Baiting experiments with oospores of the oomycetes parasites *Phytophthora megasperma, P. cactorum, Pythium* sp. and *Aphanomyces euteiches*, revealed that those baits quickly became infected by different hyperparasites (Sneh et al., 1977). Another approach for characterizing zoosporic hyperparasites would be to implement a combination of DNA and RNA isolation methods combined with specific primers and to then visualize the respective organisms using specific FISH (Fluorescence *in situ* hybridization) probes (Not et al., 2002; Jones et al., 2011; Marano et al., 2012). Such targeted molecular probing techniques are a powerful tool to identify unknown organisms. When attempting to detect hyperparasites by this approach, however, mainly free living stages (zoospores) will be detected and the sampling is largely limited to aquatic environments because the background fluorescence in soil or sediment samples tends to be high (Wagner and Haider, 2012).

Hyperparasites, their hosts and the primary hosts are complex systems. Most studies about zoosporic hyperparasites base their evidence on laboratory studies of dual cultures of one host infected by one parasite or the host range of a single parasite (e.g., Karling, 1960; Sparrow, 1960; Held, 1981). Although to date we can only estimate how those interactions might occur in natural environments like sediment or soil (Gleason et al., 2012), simultaneous infections by different species are likely—especially for abundant parasite hosts for which more than one species of hyperparasite is known (for examples see **Tables 1, 2**). Similarly, unrelated or distantly related hyperparasites may infect the same hosts individually or simultaneously. An excellent example of this phenomenon was described by Karling (1960) who observed simultaneous infection of *Rhizophlyctis rosea* with four hyperparasites. He studied infections of the facultative parasite *R. rosea* with *Chytriomyces verrucosa* (Chytridiomycota). Karling noted that numerous sporangia of *R. rosea* were also infected with *Rozella rhizophlyctii* (Rozellida/Cryptomycota) and *Olpidiopsis karlingiae* (Oomycota). In addition to this, the large sporangia of *R. rosea* were infected by a fourth species, *Pythium proliferum* (Oomycota), which was itself densely parasitized by *Woronina pythii* (Phytomyxea). Although *R. rosea* is a facultative parasite, this example shows the extent to which hyperparasites can occur in nature when studied in detail.

On the other hand not all hyperparasites are host specific. Studies on the range of host specificity indicate that some species of hyperparasites in the Oomycota and Phytomyxea can infect several species of hosts (Goldie-Smith, 1951; Dylewski and Miller, 1983). *Rozella allomycis* only infects two susceptible hosts: *Allomyces arbuscula* and *A. macrogynus* (Held, 1974), while *Olpidiopsis incrassata* infects six species of *Saprolegnia* and three species of *Isoachlya* (Slifkin, 1961). Other parasites such as *Woronina pythii* have a broad host spectrum and can infect more than 40 species of oomycetes (Dylewski and Miller, 1983). *Pythium oligandrum* also infects a wide range of fungal and stramenopilous host (Ribeiro and Butler, 1995). These studies highlight the importance of isolating and characterizing species for understanding and characterizing hyperparsite biodiversity

and host range. Culture based methods and well defined voucher isolates are also needed to provide a groundwork for DNA barcoding studies (del Campo et al., 2014) or for food web analyses (Hrcek et al., 2011) which form the basis for a more holistic understanding of hyperparasites and their ecological roles.

SIZE CONTROL OF HOST POPULATIONS BY HYPERPARASITES

Like all parasites, hyperparasites can impact population size and fitness of their hosts (Sieber and Hilker, 2011; Allen and Bokil, 2012; Preston et al., 2014). Some hyperparasites can infect persistent structures of their hosts, for example oospores, resistant sporangia, or resting spores (Gleason et al., 2010). Such resting stages are recalcitrant substrates and can survive in a dormant state in dried soil for long periods of time (Goldie-Smith, 1956b; Bruckart et al., 2011) where they accumulate, forming a "spore bank" of zoosporic parasites. But when these resting stages are infected by hyperparasites the pathogen pressure can potentially be reduced. This could explain the finding that zoosporic hyperparasites can be linked to suppressive soil properties (Weller et al., 2002) as they have the ability to reduce the viable pathogen load in soil. The presence of hyperparasites contributes to controlling their hosts in the environment, hinting at the important role of these parasites in balancing diversity and abundance of their hosts, consequently resulting in stable ecosystems.

Hyperparasites are already widely used as biological control agents to control the population size of plant pathogens. The best known example is the oomycete *Pythium oligandrum* which is used to control other *Pythium* spp. and oomycetes (Ikeda et al., 2012). Hyperparasites have a huge potential to control diseases if they can be systematically accumulated in the environment. But so far not many hyperparasites can be grown in the lab in big enough quantities that permit use as biocontrol agent. There are known hyperparasites of important plant pathogens which have not been explored as biocontrol agents because of this reason. Oospores of the potato pathogen *Phytophthora erythroseptica*, for example, were found to be infected with *Hyphochytriun catenoides* and *Rhizidiomyces japonicus* in waterlogged soils in England (Wynn and Epton, 1979). Given the global importance of *Phytophthora* spp. as existing and emerging plant pathogens (Brasier et al., 2004; Fry, 2008; Fisher et al., 2012), identifying hyperparasites that naturally control the abundance and survival of these parasites would be beneficial.

There have been observations of such effects in control of population sizes by hyperparasites in fresh water ecosystems. Populations of *Zygorhizidium affluens* (Chytridiomycota) are frequent parasites of populations of the diatom *Asterionella formosa* in freshwater lakes (Canter, 1965; Beakes et al., 1988). The growth of the parasite population follows the growth of the host population (Chave, 2013) resulting in a "chytrid epidemic." Sporangia and resting spores of *Z. affluens* can be infected by the hyperparasite *Rozella parva* (Canter, 1965). Both a decline in the *A. formosa* populations and an increase in the *R. parva* populations as the growing season progresses would, in theory, result in a decrease in *Z. affluens* populations. Another example is *Polyphagus euglenae*, a parasite of *Euglena viridis* and *E. gracilis* and its hyperparasite *Rozella polyphagi* (Powell, 1984), in which an infection with the hyperparasite *R. polyphagi* is known to decrease the population size of its host. Blooms of toxic cyanobacteria are common in freshwater environments (Sønstebø and Rohrlack, 2011). These cyanobacteria can be parasitized by zoosporic true fungi (Canter, 1972) that have the potential to control the sizes of such toxic algal blooms. Parasites of cyanobacteria can be infected by hyperparasites, a fact which was noted, but not analyzed in any detail. A reduction in the numbers of zoosporic parasites may result in an increase in growth of the (toxic) algal blooms (Canter, 1972). However, such tripartite interactions should be the subject of future studies: hyperparasites may impact the population sizes of parasitic, zoosporic true fungi that are parasites of organisms which can be damaging to the environment. The need to study the ecological role of hyperparasites may be even more significant as cyanobacteria and microalgae are gaining increasing importance as sustainable second generation biofuels (Stephens et al., 2010). Microalgal cultures are prone to get contaminated with a wide range of bacteria and eukaryotes which potentially impact on the yield (Stephens et al., 2010; Lakaniemi et al., 2012). Especially in such semi-controlled systems a control of detrimental parasites with hyperparasites could be a successful approach to increase productivity and energy yield.

FOOD WEBS

The presence of hyperparasites in food webs affect predators and grazers alike (**Figure 2**) (Hatcher et al., 2006; Morozova et al., 2007). By infecting resistant structures of their hosts, zoosporic parasites and hyperparasites release recalcitrant carbon, which is then potentially made available as food for protistan and metazoan predators rather than being deposited through sedimentation (**Figure 2D**). When zoospores are released, some will find new utilizable substrates, some will encyst, but many may provide food for grazing zooplankton and filter feeding animals (**Figure 2A**) (Kagami et al., 2007; Miki et al., 2011). The sizes of the mouth parts of grazing zooplankters determines the maximum size of zoopores that can be ingested (Kagami et al., 2007). For example, species of *Daphnia* are known to digest zoospores of any species smaller than 5 μm in diameter. The sizes of zoospores of hyperparasites tend to be smaller than those of the hosts (Sparrow, 1960; Held, 1981). This is clearly exemplified by the parasitic relationship between the fish parasite *Achlya flagellata* and its hyperparasite *Dictyomorpha dioica* (Mullins and Barksdale, 1965). The zoospores of *A. flagellata* are 8.5–10.5 μm in diameter while those of *D. dioica* are 3.5 μm in diameter (Mullins and Barksdale, 1965). The smaller size of the hyperparasite zoospores may enable zooplankton to graze on them or make their ingestion by zooplankters more likely, so that they ultimately provide better food resources for zooplankton than parasite zoospores. The population sizes of key species of grazing zooplankters, such as *Daphnia*, may be impacted by a decrease or increase in the total supply of zoospores which are a good food source (Kagami et al., 2007). This in turn will impact the population sizes of planktonivorous fish and other macroinvertebrates which feed on zooplankton.

Because of the high nutritional value of zoospores, we would expect populations of *Daphnia magna* to increase with the onset of the chytrid epidemic. *Daphnia magna* also feeds on zoospores

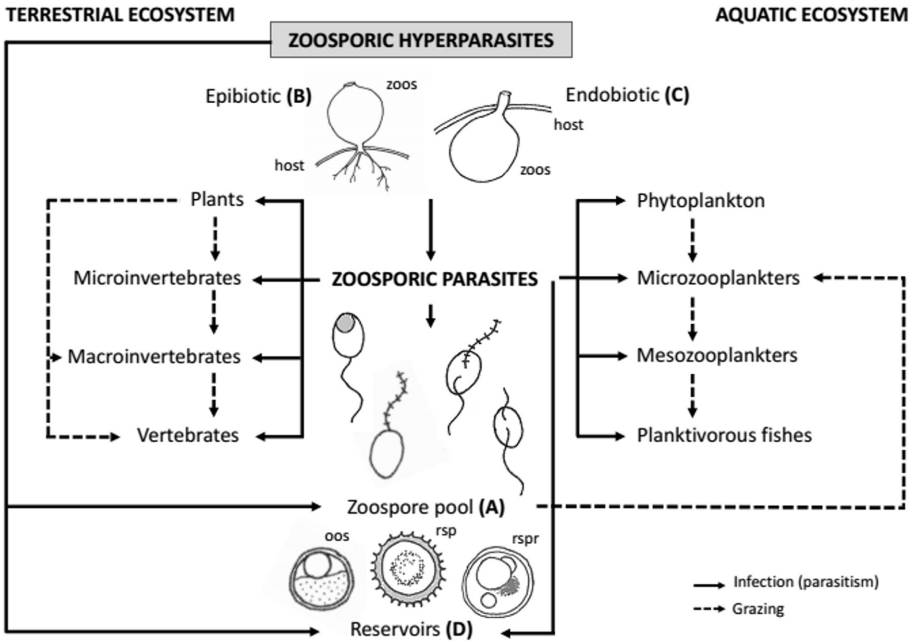

FIGURE 2 | Possible links of a hypothesized food web in which zoosporic parasites and hyperparasites are involved. In food webs zoosporic hyperparasites can either contribute the zoospore pool (Zoospore pool, **A**) which is used as food source by grazers in terrestrial and acquatic ecosystems. At the same time epibiotic sporangia of hyperparasites (Epibiotic, **B**) can serve as food source for larger grazers. The sporangia of epibiotic hyperparasites (Endobiotic, **C**) are more difficult to access as food sources for grazers. Some zoosporic hyperparasites use resting stages (Reservoirs, **D**) as substrate. Hosts of hyperparasites can be parasites of microscopic eukaryotes, but also parasites of plants or animals. This allows for a rapid cycling of nutrients from organisms higher up in the food web towards small grazers (trophic upgrading). References: zoos, zoosporangium; host, zoosporic host; oos, oospore; rsp, resting spore; rspr, resting sporangium.

of *Batrachochytrium dendrobatidis* (Chytridiomycota), which is a serious pathogen of amphibians (Buck et al., 2011). It was suggested that the consumption of zoospores of *B. dendrobatidis* by *D. magna* may prevent the transmission of this fungus (Buck et al., 2011). If a crash occurs in populations of *D. magna*, when the total zoospore food supply rapidly decreases, the rate of transmission of amphibian chytridiomycosis could increase because fewer individuals of *D. magna* would be present to feed on zoospores of *B. dendrobatidis*. Thus, more zoospores would be available to spread chytridiomycosis through the populations of amphibians. In adult frogs *B. dendrobatidis* prevalence is highest during late summer and winter, while infection takes place from late spring to early summer (Russell et al., 2010; Sapsford et al., 2013). This coincides with the breakdown of the chytrid epidemics. We would expect many other biotic and abiotic factors to affect population dynamics here, but the availability of zoospores as food in the spring can be decisive for the pathogen load of *B. dendrobatidis* later in the year by influencing the numbers of predators feeding on zoospores.

It is important to establish the roles of zoosporic hyperparasites as well as parasites in the structure and function of aquatic food webs. Structure includes species richness, trophic levels, links, trophic chain length, and connectance (Dunne et al., 2005, 2013). Function includes the total amount, rate, and efficiency of carbon transfer, and effects on stability of the food web. Adding parasites to food webs results in an increased complexity (Lafferty et al., 2008; Thieltges et al., 2013). Adding links to food webs, such as parasites, hyperparasites, and both of their associated niches, might also add to the stability of a particular web (Hudson et al., 2006; Lafferty et al., 2006, 2008). Parasites with life cycles involving ontogenetic niche shifts—such as hyperparasites—impact food web structures more and potentially negatively because specialized life cycle stages are more prone to secondary extinction than generalist stages (Preston et al., 2014). Such ontogenetic effects can be found in zoosporic hyperparasites: different types of zoospores, or zoospores formed by different species can have considerably different swimming patterns (Lange and Olson, 1983) or serve different purposes like long or short distance dispersal (Neuhauser et al., 2011a). Consequently different zoospores will attract predators occupying different niches and will therefore enter the food web at different trophic levels. Because of the anecdotal nature of the available data it is not yet possible to include zoosporic hyperparasites into mathematical food web models to allow for more realistic estimates of population dynamics and energy flow and their impact on food web stability. However, it can be expected that once our knowledge about zoosporic hyperparasites increases, we will also be able to show that, like zoosporic true fungi, zoosporic hyperparasites are diverse, abundant, and important links for energy transfer (Grami et al., 2011; Niquil et al., 2011). Zoosporic true fungal parasites result in a significant reduction in the loss of algal carbon though sedimentation into the detritus pool, allowing carbon transfer from zoospores to grazing protists and metazoans. This contributes to longer carbon path lengths, higher levels of activity and specialization, lower recycling, and increased stability of aquatic food webs (Grami et al., 2011; Ulanowicz et al., 2014).

Hyperparasites tend to have shorter life cycles than their hosts, so they produce biomass in the form of zoospores more quickly. Some of them produce primarily zoospores, such as *Rozella*, which, instead of forming its own zoosporangium, uses the host sporangium to reproduce (Held, 1981; Powell, 1984). This outsourcing of energy consuming biomass production allows for faster life cycles and hyperparasites such as *Rozella* are therefore likely to increase and accelerate the energy flow between trophic levels (**Figure 2C**). On the other hand epibiotic parasites have zoosporangia that are formed on the surface of their host. Consequently, both their zoospores and the zoosporangia are likely to enter the food web contributing different types of energy for predators with different size preferences for their food (**Figure 2B**). Since food webs that include zoosporic hyperparasites have additional links, we suggest they could be more efficient, and therefore would support a larger population of grazing zooplankton species. This hypotheses needs to be tested quantitatively.

CONCLUSION AND FUTURE PROSPECTS

Many hyperparasites have been discovered during research with the host species. However, it is vital that such efforts are intensified to provide the basis for the development of more rapid tools for species discovery and characterization. Although emerging techniques such as single cell genomic approaches provide a quantum leap in identifying and characterizing active cells in the environment, such methods will initially not account for the complex life cycles of zoosporic hyperparasites. To understand the life cycles, and consequently the ecological function of hyperparasites, time consuming studies involving targeted sampling and probing approaches are still needed. Even the sparse information available on hyperparasites highlights their potential in many ecosystem processes. Zoosporic hyperparasites may increase the turn-around time of certain nutrients in food webs due to their often rapid life cycles. They may play a role in trophic upgrading, as well as in the stability and complexity of food web dynamics. Hyperparasites also may play a role in the natural regulation of their host population sizes, which are also parasites. Regulation of population sizes of parasites will have an impact on their host population sizes. This may result in fine-tuning the magnitudes of patterns of energy flow in food webs and impact overall biodiversity as well as population dynamics. In summary, it is likely that zoosporic hyperparasites play a vital part of every ecosystem; hence more focused research on these important organisms is needed.

AUTHOR CONTRIBUTIONS

Frank H. Gleason and Sigrid Neuhauser drafted the initial version of the manuscript. Agostina V. Marano, Télesphore Sime-Ngando, Martin Kirchmair, Brooke K. Sullivan and Osu Lilje critically revised this draft and contributed intellectual content to the final version.

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Current ecological understanding of fungal-like pathogens of fish: what lies beneath?

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Despite increasingly sophisticated microbiological techniques, and long after the first discovery of microbes, basic knowledge is still lacking to fully appreciate the ecological importance of microbial parasites in fish. This is likely due to the nature of their habitats as many species of fish suffer from living beneath turbid water away from easy recording. However, fishes represent key ecosystem services for millions of people around the world and the absence of a functional ecological understanding of viruses, prokaryotes, and small eukaryotes in the maintenance of fish populations and of their diversity represents an inherent barrier to aquatic conservation and food security. Among recent emerging infectious diseases responsible for severe population declines in plant and animal taxa, fungal and fungal-like microbes have emerged as significant contributors. Here, we review the current knowledge gaps of fungal and fungal-like parasites and pathogens in fish and put them into an ecological perspective with direct implications for the monitoring of fungal fish pathogens in the wild, their phylogeography as well as their associated ecological impact on fish populations. With increasing fish movement around the world for farming, releases into the wild for sport fishing and human-driven habitat changes, it is expected, along with improved environmental monitoring of fungal and fungal-like infections, that the full extent of the impact of these pathogens on wild fish populations will soon emerge as a major threat to freshwater biodiversity.

Keywords: emerging infectious disease, aquatic, extinction, vertebrate, global, biodiversity, Oomycota, Mesomycetozoea

INTRODUCTION

Fishes are susceptible to diseases caused by a large number of infectious agents including viruses, bacteria, true fungi, fungal-like microrganisms, other protists, and metazoans. This review will briefly discuss true fungal pathogens and then focus on commonly reported zoosporic and amoeboid fungal-like pathogens in the Oomycota and Mesomycetozoea. In general, the number of reported fungal and fungal-like pathogens responsible for diseases in animals is on the increase globally (Fisher et al., 2009; Holdich et al., 2009; Loo, 2009; Frick et al., 2010; Ratnieks and Carreck, 2010; Sarmiento-Ramírez et al., 2010). As such, they are truly emerging diseases with increasing incidence, geographic range, virulence, and some of these fungal and fungal-like pathogens have recently been found in new hosts or are newly discovered (Berger et al., 1998; Brown, 2000; Daszak et al., 2000; Kim and Harvell, 2004; Blehert et al., 2009; Peeler et al., 2010; Cameron et al., 2011). The underpinning drivers of this observed increase remain unclear but these pathogens are known to be opportunistic (Fisher et al., 2012), to have resilient and relatively long-lived environmental stages (Mitchell et al., 2008; Andreou et al., 2009) and may have benefited from recent increase in global trade (Brasier, 2008) and spread of invasive species (Gozlan et al., 2010). Thus increasingly infectious outbreaks are reported in a broad range of species from coral (Kim and Harvell, 2004) to wheat (Wanyera et al., 2006); notable examples include local extinctions of bats (Frick et al., 2010), bees (Ratnieks and Carreck, 2010), turtles (Sarmiento-Ramírez et al., 2010), amphibians (Fisher et al., 2009) and fish (Gozlan et al., 2005, 2009). In aquatic ecosystems fungi and fungal-like pathogen detection in fish hosts is more complicated due to the lack of direct observation of their hosts contrary to frogs or coral, for example (Gozlan, 2012). This is particularly true in freshwater systems where, despite being responsible for pan-continental population extinctions, some diseases caused by fungal and fungal-like pathogens are chronic with no clear external symptoms (Gozlan et al., 2005; Kocan and Hershberger, 2006; Andreou et al., 2011, 2012). This is very well illustrated, for example, by the rosette agent *Sphaerothecum destruens*, which has been rapidly spreading all over Europe via an invasive healthy fish host carrier (Gozlan et al., 2005; Gozlan, 2012). This fungal—like pathogen is intracellular, causing high mortality (up to 90%) after about 20–30 days but it can only be confidently detected by PCR analysis (Mendonca and Arkush, 2004). The paradox is that despite huge pan-continental population extinction, it remains difficult to

characterize the true ecological impact of fungal and fungal-like pathogens on freshwater fish populations.

Despite fisheries representing a key ecosystem service for millions of people around the world, the full appreciation of disease risk associated with fungal pathogen emergence remains limited (Gozlan et al., 2006). Here, we review the current knowledge gaps of fungal microbes in fish, their phylogeography along with the current methods of detection and associated limitations and a global ecological understanding of their impacts on fish host populations. With an increasing volume of fish translocation around the world for farming and sport fishing, the relative absence of fish-infecting fungi outbreaks when compared to other more easily observed taxa exemplifies the current concern of a reporting bias in wild fish populations (Gozlan, 2012).

DIVERSITY AND PHYLOGENY

In recent years interest in the phylogeny of eukaryotes has been re-evaluated (see Adl et al., 2005). Based on data from sequencing genes, particularly rRNA gene sequences, Baldauf (2003) reassigned eukaryotes into eight different branches or supergroups within the tree of life, namely the opisthokonts, amoebozoa, plants, cercozoa, alveolates, heterokonts, discicristates, and excavates. The true fungi and Mesomycetozoea are placed along with the animals in the Opistokonta. The Mesomycetozoea form a clade, which falls on the animal branch, near the animal fungal divide (Paps et al., 2013). All of the Oomycota are placed into the Heterokont supergroup.

True fungi constitute the most species rich group of organisms on earth with 35 recognized classes and 129 orders (Hibbett et al., 2007). The majority of the fungi causing infection in fish belong to the phylum Ascomycota, with thick-walled non-motile spores (Hibbett et al., 2007). Within the Ascomycota, species from several genera have been reported to be associated with fish infections (**Table 1**). In addition to the Ascomycota, species belonging to the (earlier diverging) Zygomycota have also been reported to cause disease. The majority of the fungi, which can cause infection in fish are opportunistic and not exclusive parasites of fish. They are most commonly known as plant pathogens (e.g., *Penicillium corylophilum* and *Phoma herbarum*), soil fungi (e.g., *Paecilomyces lilacinus*) and some have even been reported to cause infection in immunosuppressed humans (e.g., *Exaphiala xenobiota* and *Ochroconis humicola*).

Branching close to the divergence between fungi and animals there is a relatively recently recognized clade of organisms, the Mesomycetozoea (Mendoza et al., 2002; Ragan et al., 1996), which includes a number of species that are pathogenic to aquatic organisms including fish (Mendoza et al., 2002; Glockling et al., 2013). Within the Mesomycetozoea, species can be divided further into the orders of Dermocystida and Ichthyophonida. The Dermocystida include a number of species that can be pathogenic to fish, the most notable being *Sphaerothecum destruens,* which can infect a wide range of hosts and has been shown to cause disease and high mortality in cyprinids (Andreou et al., 2011, 2012) and salmonid species (Arkush et al., 1998; Paley et al., 2012). The order also includes numerous *Dermocystidium* sp., which can infect a variety of fish species (see **Table 1**). The diversity of the *Dermocystidium* genus is probably underestimated as a large proportion of recorded cases in the literature only identify the pathogen to genus level. This can be addressed by applying molecular techniques to identify species. Within the Ichthyophonida, *Ichthyophonus hoferi* is the most common parasite of salt and freshwater fish (Hershberger et al., 2010; Kocan et al., 2010; Gregg et al., 2012; Hamazaki et al., 2013).

The Oomycete parasites of fishes are placed in the Phylum Oomycota and fall into either the saprolegnialean lineage or the peronosporalean lineage. The Oomycetes are water moulds which morphologically resemble fungi, but are taxonomically distinct, encompassing species that are parasitic to a large diversity of host species (Beakes et al., 2012). The majority of the species, which can infect and cause disease in fish belong to the order of Saprolegniales and fall within the genera of *Saprolegnia*, *Aphanomyces* and *Achlya*. A smaller number of species fall within the genus *Pythium*, a member of the peronsporalean lineage. Twelve species of *Saprolegnia* and six species each of *Aphanomyces* and *Achlya* (**Table 1**) are more often described in the literature as causing infection in fish; with the most common pathogens of fish being *Saprolegnia parasitica* and *Aphanomyces invadans* which have relatively high generalist indices (See **Table 1**). *S. parasitica* has been reported to cause disease in 12 fish species whilst *A. invadans* can parasitize 48 fish species.

HOST SPECIFICITY

A common characteristic of the fish pathogens within Fungi, Mesomycetozoea, and Oomycetes is their generalist nature, with the majority of species infecting and causing disease in fishes across different families (**Table 1**). All three groups include an equal proportion of species with generalist indices above 3 indicating that they are true generalists (Poulin and Mouillot, 2003). Due to higher reporting and detection of disease in farmed environments, most disease reports are from aquaculture facilities and involve cultured fish species. There is thus a bias in the fish species reported as susceptible to these pathogens and a possible underestimation of their generalist nature (Ramaiah, 2006). A large number of species have a single record of affecting a single fish species in the literature and thus the generalist index cannot be calculated.

The ability of fungal and fungal-like pathogens to infect multiple hosts ("the widest spectrum of host ranges for any group of pathogens" according to Fisher et al., 2012; see **Table 1** for fish). often drives high virulence in the most susceptible hosts (Andreou et al., 2012; Huchzermeyer and Van der Waal, 2012). The aspect of generalism in pathogenicity is important due to the fact that generalist pathogens are more likely to emerge through host switching (Woolhouse and Gowtage-Sequeria, 2005), and it is often overlooked (Yamamoto and Kilistoff, 1979; Peeler et al., 2010). However, it is commonly accepted (Ewald, 1994) that in single hosts the optimum level of virulence is determined by the trade-off between virulence and transmissibility (Davies et al., 2001). Thus, the composition of the community and the susceptibility of each host could alter its potential transmissibility and the outcome of infection (Woolhouse et al., 2001). Experimental challenges to fungal and fungal-like pathogens of several fish host species are currently needed. This could involve simple one host – one pathogen challenges such as in Andreou et al. (2012) or a

Table 1 | List of Fungi, Mesomycetozoea and Oomycetes species, which have been recorded as fish parasites in the Web of Knowledge since 1997.

Species	Order	Reported hosts	Generalist index	References
FUNGI				
Cladosporium sphaerospermum	Capnodiales	*Lutjanus campechanu*	NA	Blaylock et al., 2001
Exophiala angulospora	Chaetothyriales	*Gadus morhua*	NA	Gjessing et al., 2011
Exophiala pisciphila	Chaetothyriales	*Stegostoma fasciatum*	NA	Marancik et al., 2011
Exophiala xenobiotica	Chaetothyriales	*Pseudocaranx dentex*	NA	Munchan et al., 2009
Paecilomyces lilacinus	Eurotiales	*Clarias gariepinus* *Oreochromis niloticus niloticus* *Tilapia aurea*	2	Rand et al., 2000a,b; Ali et al., 2011
Penicillium corylophilum	Eurotiales	*Lutjanus campechanus*	NA	Blaylock et al., 2001
Ochroconis humicola	Incertae sedis	*Pseudocaranx dentex* *Pagrus major* *Sebastiscus marmoratus*	3.3	Wada et al., 2005; Munchan et al., 2009
Mucor circinelloides	Mucorales	*Pelteobagrus fulvidraco* *Pseudocaranx dentex*	5	Ke et al., 2010; Marancik et al., 2011
Phoma herbarum	Pleosporales	*Clarias gariepinus* *Oncorhynchus tshawytscha* *Oreochromis niloticus niloticus*	4	Faisal et al., 2007; Ali et al., 2011
Phialemonium dimorphosporum	Sordariales	*Mugil cephalus*	NA	Sosa et al., 2007a,b
Ochroconis humicola	Incertae sedis	*Pseudocaranx dentex* *Pagrus major* *Sebastiscus marmoratus*	3.3	Wada et al., 2005; Munchan et al., 2009
MESOMYCETOZOEA				
Dermocystidium cyprini	Dermocystida	*fluviatilis* *Gymnocephalus cernuus* *Cyprinus carpio*	3.3	Lotman et al., 2000; Pekkarinen and Lotman, 2003
Dermocystidium fennicum	Dermocystida	*PercaPerca fluviatilis*	NA	Pekkarinen and Lotman, 2003
Dermocystidium koi	Dermocystida	*Cyprinus carpio*	NA	Gjurcevic et al., 2008
Dermocystidium percae	Dermocystida	*Perca fluviatilis*	NA	Morley et al., 2008
Dermocystidium branchiale	Dermocystida	*Salvelinus alpinus* *Salmo trutta*	2	Kristmundsson and Richter, 2009
Sphaerothecum destruens	Dermocystida	*Abramis brama* *Cyprinus carpio* *Leucaspius delineatus* *Oncorhynchus kisutch* *Oncorhynchus mykiss* *Oncorhynchus tshawytscha* *Pseudorasbora parva* *Rutilus rutilus* *Salmo salar* *Salmo trutta* *Salvelinus fontinalis*	3.6	Arkush et al., 1998; Gozlan et al., 2005; Andreou et al., 2012; Paley et al., 2012
Ichthyophonus hoferi	Ichthyophonida	*Citharichthys stigmaeus* *Clupea harengus* *Clupea pallasi* *Hypomesus pretiosus* *Microgadus proximus* *Oncorhynchus kisutch* *Oncorhynchus mykiss* *Oncorhynchus tshawytscha* *Pleuronectes flesus* *Salmo trutta* *Sebastes alutus* *Sebastes emphaeus* *Sebastes flavidus* *Sprattus sprattus*	3.6	Rahimian, 1998; Criscione et al., 2002; Hershberger et al., 2002; Schmidt-Posthaus and Wahli, 2002; Gavryuseva, 2007; Kocan et al., 2010; Kramer-Schadt et al., 2010; Rasmussen et al., 2010; Gregg et al., 2012; Hamazaki et al., 2013

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Ichthyophonus irregularis	Ichthyophonida	*Limanda ferruginea*	NA	Rand et al., 2000a,b
OOMYCETES				
Achlya bisexualis	Saprolegniales	*Mugil cephalus*	NA	Sosa et al., 2007a
Achlya klebsiana	Saprolegniales	*Oreochromis niloticus niloticus*	2.3	Ali et al., 2011; Cao et al., 2013
		Clarias gariepinus		
		Pelteobagrus fuvidraco		
Achlya americana	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Achlya. oblongata	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Achlya racemosa	Saprolegniales	*Odonthestes bonariensis*	NA	Pacheco Marino et al., 2009
Achlya ambisexualis	Saprolegniales	*Oncorhynchus mykiss*	NA	Vega-Ramirez et al., 2013
Aphanomyces parasiticus	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Aphanomyces frigidophilus	Saprolegniales	*Coregonus lavaretus holsatus*	2	Czeczuga et al., 2004, 2005
		Salmo trutta		
Aphanomyces invadans	Saprolegniales	*Alosa sapidissima*		
		Anguilla anguilla		
		Ameiurus melas		
		Archosargus probatocephalus		
		Bairdiella chrysoura		
		Brevoortia tyrannus		
		Brycinus lateralis		
		Barbus poechii		
		Barbus paludinosus		
		Barbus unitaeniatus		
		Catla catla		
		Channa marulius		
		Clarias gariepinus		
		Clarias ngamensis		
		Cyprinus carpio		
		Fundulus heteroclitus		
		Fundulus majalis		
		Hepsetus odoe		
		Hydrocynus vittatus		
		Ictalurus punctatus		
		Leiopotherapon unicolor		
		Labeo lunatus		
		Labeo cylindricus		
		Lepomis macrochirus		
		Macquaria ambigua		
		Maccullochella peelii		
		Marcusenius macrolepidotus		
		Micralestes acutidens		
		Micropterus salmoides		
		Mugil cephalus		
		Mugil curema		
		Nematalosa erebi		
		Oncorhynchus mykiss		
		Oreochromis andersonii		
		Oreochromis macrochir		
		Petrocephalus catostoma		
		Pharyngochromis acuticeps		
		Pogonias cromis		
		Sargochromis codringtonii		
		Sargochromis giardi		
		Serranochromis robustus		
		Serranochromis angusticeps		
		Serranochromis macrocephalus		
		Schilbe intermedius		
		Silurus glanis		
		Tilapia sparrmanii		

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Tilapia rendalli		*Trinectus maculates*	3.7	Thompson et al., 1999; Hawke et al., 2003; Harikrishnan et al., 2005; Kiryu et al., 2005; Webb et al., 2005; Vandersea et al., 2006; Sosa et al., 2007b; Oidtmann et al., 2008; Saylor et al., 2010; Boys et al., 2012; Go et al., 2012; Huchzermeyer and Van der Waal, 2012; Saikia and Kamilya, 2012
Aphanomyces irregularis	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Aphanomyces laevis	Saprolegniales	*Aplocheilus panchax* *Clarias gariepinus* *Oreochromis niloticus niloticus*	4	Mondal and De, 2002; Ali et al., 2011
Aphanomyces salsuginosus	Saprolegniales	*Salangichthys microdon*	NA	Takuma et al., 2010
Saprolegnia australis	Saprolegniales	*Oncorhynchus nerka* *Plecoglossus altivelis* *Salmo trutta*	3.3	Hussein et al., 2001; Chang et al., 2002; Fregeneda-Grandes et al., 2007
Saprolegnia brachydanis	Saprolegniales	*Danio rerio*	NA	Ke et al., 2009a,b
Saprolegnia diclina	Saprolegniales	*Acipencer persicus* *Oncorhynchus mykiss Salmo salar* eggs *Salmo trutta* *Sciaenops ocellatus*	3.3	Leano et al., 1999; Fregeneda-Grandes et al., 2007; Ghiasi et al., 2010; Shahbazian et al., 2010; Thoen et al., 2011
Saprolegnia ferax	Saprolegniales	*Carassiuus auratus* *Coregonus lavaretus holsatus* *Odonthestes bonariensis* *Oncorhynchus mykiss* eggs *Salmo trutta*	3.6	Czeczuga et al., 2004; Fregeneda-Grandes et al., 2007; Ke et al., 2009a,b; Pacheco Marino et al., 2009; Shahbazian et al., 2010; Cao et al., 2013
Saprolegnia furcata	Saprolegniales	*Salmo trutta*	NA	Fregeneda-Grandes et al., 2007
Saprolegnia hypogyana		*Oncorhynchus mykiss* eggs *Salmo trutta*	2	Fregeneda-Grandes et al., 2007; Shahbazian et al., 2010
Saprolegnia parasitica	Saprolegniales	*Acipencer persicus* *Astyanax eigenmanniorum* *Astyanax fasciatus* *Bidyanus bidyanus* *Coregonus lavaretus holsatus* *Ictalurus punctatus* *Odontesthes bonariensis* *Oncorhynchus mykiss* *Oncorhynchus masu*		
		eggs *Oncorhynchus nerka* *Salmo salar* eggs *Salmo trutta* *Salvelinus leucomaenis*	3.3	Bangyeekhun et al., 2001; Hussein and Hatai, 2002; Czeczuga et al., 2004; Fregeneda-Grandes et al., 2007; Mancini et al., 2008, 2010; Mifsud and Rowland, 2008; Ghiasi et al., 2010; Shahbazian et al., 2010; Thoen et al., 2011

(Continued)

Table 1 | Continued

Species	Order	Reported hosts	Generalist index	References
Saprolegnia polymorpha	Saprolegniales	*Cyprinus carpio*	NA	Willoughby, 1998
Saprolegnia salmonis	Saprolegniales	*Coregonus lavaretus holsatus* *Oncorhynchus masu* *Oncorhynchus mykiss* *Oncorhynchus nerka* *Plecoglossus altivelis* *Salmo trutta* *Salvelinus leucomaenis*	2.4	Hussein et al., 2001; Chang et al., 2002; Hussein and Hatai, 2002; Czeczuga et al., 2004, 2005
Saprolegnia shikotsuensis	Saprolegniales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2005
Pythium aquatile	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium pulchrum	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium thalassium	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004
Pythium torulosum	Pythiales	*Coregonus lavaretus holsatus*	NA	Czeczuga et al., 2004

A generalist index was calculated for each parasite using the method described in Poulin and Mouillot (2003); where species with two or more hosts can have generalist indices ranging from 1 (all host species share the same genus) to 5, using the five taxonomic levels of genus, family, order, class, and phylum. NA stands for non-applicable as the index cannot be calculated when only one host has been reported. The fish taxonomy proposed by Nelson (1994) was used in calculating all generalist indices.

combination of multi-hosts challenges. In addition, experimental data on the free-living elements of these life cycles of pathogens such as the presence of zoospores, would allow the measurement of their production, longevity in the system and their resistance to a range of abiotic factors (e.g., temperature, PH). These data are needed to build reliable models to test host susceptibility, understand the controlling factors of infectious phase as well as the recovery phase typical of SIT or SEIR epidemiological models (susceptible-exposed-infectious-recovered).

LIFE CYCLES AND STAGES

In the assimilative phases of oomycetes and most of the true fungi, colonization of new tissues is accomplished through the growth of hyphae, with the exception of the black yeasts, *Exophiala*, which may transition between yeast and hyphal forms (dimorphism) (de Hoog et al., 2011). Mesomycetozoeans more often grow as round multinucleate coenocytes. These can be concentrated in visible cysts in the genus *Dermocystidium* (e.g., Lotman et al., 2000) or disseminated or nodular in *S. destruens* and *Ichthyophonus* (Sindermann and Scattergood, 1954; Arkush et al., 1998). Hyphal forms have been described in some *Dermocystidium* (Dykova and Lom, 1992) and are common in *Ichthyophonus* (Sindermann and Scattergood, 1954; Rand, 1994; Franco-Sierra and Alvarez-Pellitero, 1999). Only true fungi, however, have septate hyphae, although some oomycetes have segmented or plugged thalli and thus are also compartmentalized.

All fungal and fungal-like pathogens have prolific asexual reproduction (r-strategy) functioning for dispersal or further dissemination within the host. In the fungi, this is through the production of conidiospores (Ascomycota) or sporangiospores (Zygomycota), and the budding of yeast stages. These spores are not motile and are protected by a chitinous cell wall. The durability and resilience of these spores is an important adaptation for increasing opportunities to encounter new susceptible hosts (Fisher et al., 2012). These spores can survive in a dormant state during conditions unfavorable for growth. Oomycetes produce biflagellated zoospores within sporangia, usually located either at the terminal ends of hyphae. These spores function to disperse the parasite between hosts and typically encyst after a short period of motility. In *Saprolegnia* species longer lived secondary zoospores emerge from cysts produced by primary zoospores. This pattern of re-emergence called polyplanetism, may be repeated several times (Bruno et al., 2011), and most likely functions to allow several opportunities to contact a new host. Zoospores of many oomycetes are chemotactic, responding to amino acids, carbohydrates and a range of aldehyde attractants (Donaldson and Deacon, 1993). The encysted zoospores of *S. parasitica* are decorated by long hooked hairs that are thought to aid in attachment to the fish host (Van West, 2006; Walker and Van West, 2007).

Reproduction in the Mesomycetozoea is more varied (Mendoza et al., 2002). *S. destruens* produce non-motile walled endospores which may either infect other cells within the same host or spread and infect a new host (Arkush et al., 2003). Endospores also produce singly flagellate zoospores upon exposure to fresh water (Arkush et al., 2003) but it is not clear whether these zoospores are infective (Paley et al., 2012). *Dermocystidium* has similar development with zoospore development within spores, but zoospores are infective (Olson et al., 1991). Released endospores of both *Dermocystidium salmonis* and *S. destruens* have the capacity to release zoospores for several weeks at 4°C (Olson et al., 1991; Andreou et al., 2009). The life cycle of *Ichthyophonus* is less understood and varies with pH (Okamoto et al., 1985; Spanggaard et al., 1995; Franco-Sierra and Alvarez-Pellitero, 1999). Single and multinucleate endospores are produced in culture and *in vivo* (Okamoto et al., 1985; Spanggaard et al., 1995; Franco-Sierra and Alvarez-Pellitero, 1999). Motile zoospores are not produced but amoeboid stages are released under specific pH optima in culture (e.g., Okamoto et al., 1985). Transmission is also not well understood, except

that the parasite can be acquired through carnivory (Jones and Dawe, 2002). Kocan et al. (2013) describe small amoeboid stages within the stomach wall of sculpin and trout hosts after feeding of infected tissues and hypothesize that these amoebae represent the infectious stage. The infectious stage of planktivorous fish is still unknown (Gregg et al., 2012) and an alternate host is suspected (Sindermann and Scattergood, 1954).

In parasites of fishes sexual reproduction (s-strategy) has only been described in a few oomycetes. When sexual reproduction occurs, the two dissimilar gametangial structures called the oogonium and the antheridium grow closer together until they fuse, and haploid nuclei from the antheridia fertilize the eggs within the oogonia forming diploid oospores. In free living oomycetes the fertilized zygote, or oospore, is typically resistant and can survive for prolonged periods. Meiosis and recombination occur before germination of the oospore. However, the main oomycete pathogens of live fish (e.g., *A. invadans* and *S. parasitica*), do not generally (in case of *A. invadans* never) reproduce sexually and therefore rely entirely on asexual zoosporogenesis (r-strategy). Some egg infecting species do produce oogonia (e.g., *S. australis*, *S. diclina*, *S. ferax*) but even in these species oospore germination is rarely if ever observed. It is highly unlikely that oospores serve as effective resistant survival structures for fish parasitic oomycetes. Most true fungal parasites of fish are described as "fungi imperfecti," based on the lack of a described sexual stage.

TROPHIC MODES

Research on animal parasites has revealed that many of these species are not exclusively saprophytic or parasitic (Gleason et al., 2010; McCreadie et al., 2011). In fact, their precise ecological functions can only be understood with intensive metagenomic investigations, which have rarely been conducted (Jiang et al., 2013). Nonetheless, these microorganisms are frequently characterized as either saprotrophs or biotrophs (Gleason et al., 2010; McCreadie et al., 2011). Saprotrophs usually do not infect live hosts, rather they grow on non-living organic material. In contrast many biotrophs cannot grow outside the host, but some can be grown in culture. Growth of parasites in culture allows research on mechanisms of infection and sequencing genes. Facultative parasites can grow well as either parasites or saprotrophs. Many eukaryotic microorganisms are thought to be parasites primarily because they cannot be grown outside their host, but in fact their trophic relationships remain to be determined. Many Oomycete species are primarily saprotrophs, yet few can become parasites under certain conditions, such as compromised immunity in their hosts. The important point is that they have alternative substrates for growth outside the host, which is an important characteristic of emerging infectious diseases (EID) (Fisher et al., 2012).

PROTEINS AS SUBSTRATES FOR GROWTH

For a long time proteins have been known to be good substrates for the isolation of Oomycetes into pure culture and for their subsequent growth in liquid media (Sparrow, 1960). For example, casein and keratin can be useful substrates for isolation and growth. Furthermore animal hosts and tissues are known to be protein rich environments. Czeczuga et al. (2002) isolated many species of Oomycetes from specimens of fish muscles placed in freshwater lakes. Some of these specimens came from fish, which were known to be hosts for Oomycetes. Smith et al. (1994) demonstrated proteolytic activity of *Saprolegnia diclina, ferax,* and *parasitica* by observing the clearing of casein on solid media. Proteins must be digested extra-cellularly and the amino acids produced must be transported into the cell prior to their catabolism. Jiang et al. (2013) documented the presence of genes for serine, metallo- and cysteine proteases and genes for amino acid transporters in the complete sequence of the genome of *S. parasitica*.

Saprotrophic isolates of *Saprolegnia*, *Achlya*, *Dictyuchus*, *Leptolegnia*, *Aphanomyces*, *Apodachlya,* and *Pythium* grew rapidly on many but not all amino acids as sole sources of carbon and nitrogen in liquid media (Gleason et al., 1970a,b; Faro, 1971). Alanine, proline, glutamate, aspartate, leucine, lysine, arginine, serine, and phenylalanine were especially good carbon sources, there was very little or no growth on valine, isoleucine, threonine, methionine, and glycine, and there were considerable differences in rates of utilization among the species tested. Saprotrohic and parasitic isolates of *Saprolegnia* can remove all amino acids from liquid media during growth on mixtures of amino acids (Gleason, 1973; Nolan, 1976). These data indicate that many Oomycetes have the capacity for digestion of proteins and subsequent uptake and catabolism of amino acids. Therefore they commonly grow in protein rich environments. Recently, a few species in the Mesomycetozoea have been grown in culture (Glockling et al., 2013), but nutritional experiments have not been conducted, and little is known about their proteolytic capacities.

CURRENT DETECTION TECHNIQUES

Lesions formed by parasites were initially characterized from phenotypic, serological and morphological properties of the pathogen. Isolation and culturing of causative organisms from swabbed lesions of infected fish has been an integral part in understanding the taxonomic groupings, etiology of the disease, infectivity, and host-parasite relationships. The process of isolating and identifying pathogens can however be a time consuming process requiring a high level of technical expertise.

Morphological identification of microbial species, which often requires identification of reproductive stages, is difficult to accomplish directly from ulcerated tissue. Direct visualization of pathogens in infected tissues has been made possible with the development of species-specific fluorescent probes. For example, the monoclonal antibody MAb 3gJC9, which is specific for an antigen involved in the pathogenicity of *Aphanomyces astaci* and *A. invadans (=piscicida)* in infected crayfish and fish respectively, has been used for immunofluorescent identification of these species in infected tissues (Miles et al., 2003). The approach was found to be more sensitive than the conventional staining method, Grocott's methanamine silver stain, in that it enabled the detection of early stages of infection (Grocott, 1955; Miles et al., 2003). Fluorescent hybridization (FISH) probes have also been used to identify specific pathogens in infected tissues *in situ*. For example, *A. invadans* was found to be a primary oomycete pathogen in ulcerative mycosis of infected estuarine fish in North Carolina and Florida using a FISH assay (Vandersea et al., 2006; Sosa et al., 2007a,b). Continuing improvements in

isolation, culturing and *in situ* approaches is essential for broadening our understanding of disease pathology and etiology and more fundamentally the morphology and physiology of these pathogenic species. In comparison to morphological and physiological classification, the rapid advances in molecular techniques has improved the reliability and accuracy of the tool in distinguishing many taxa, such as the microsporidian taxa (Larsson, 2005). A molecular approach has also led to rapid development of diagnostic tools which involve polymerase chain reaction (PCR), amplification of nucleic acids, restriction enzyme digestion, probe hybridization and nucleotide sequencing. The development of the FISH assay for example was as a result of using a sensitive PCR technique. The use of PCR to detect and identify infections has become commonplace (Tsui et al., 2011). A large number of disease-causing pathogens are often identified to genus level (e.g., *Dermocystidium* sp.) and not species level. The number of species being identified has been constantly increasing through the use of molecular tools for disease detection and identification. A concerted effort to use the same DNA loci would increase the available genetic information resulting in a better resolution of the phylogenetic relationships within and between these groups. The 18S rRNA gene has been used extensively (for Fungi and Mesomycetozoans); however the Internal Transcribed Sequence 1 (ITS1) has been more extensively used within the Oomycetes. As documented by Diéguez-Uribeondo et al. (2007) for the *S. diclina- S. parasitica* complex, both molecular and morphological and physiological data can help solve phylogenetic relationships. Thus, using ITS rRNA gene, five phylogenetic separate clades were identified for the *Saprolegnia* complex, with all isolates collected from salmonid lesions falling into a single clade (i.e., clade I). However, within that clade I, parasitic isolates came from a wide range of hosts including, for example, crustaceans, and catfish but also non-pathogenic isolates from soil and water. Molecular analyses have the potential to discriminate at the subspecies or strain level (Phadee et al., 2004). The level of sensitivity of the molecular techniques in the clinical context has however been sporadic (Cunningham, 2002). This is largely due to the relatively low genomic information that is available through public data bases such as Genbank. We propose that all reported cases of disease outbreaks should have both of these regions sequenced and reported within the literature. The use of these loci will allow both detection at species level (18S rRNA) and identification of different strains (using ITS1) within the same species; allowing for a better identification and detection of virulent strains. This collection of information alongside morphological and physiological data will increase the resolution of the phylogenetic information and the sensitivity of molecular identification.

Alternative detection approaches include loop-mediated isothermal amplification (LAMP) and pyrolysis mass spectrometry. LAMP has the potential of increasing sensitivity of pathogen compared to PCR and unlike PCR it is not inactivated by tissue and blood-derived inhibitors or genomic DNA (Savan et al., 2005). It has been used in the detection of trypanosome infection (Savan et al., 2005). Pyrolysis mass spectrometry profile and canonical variate analysis have been used to demonstrate clusters of *A. invadans* isolates and discriminate them from non-pathogenic *Aphanomyces* species (Lilley et al., 2001). The development and refinement of multiple approaches of detection have their place in increasing the knowledge of the pathogen, its distribution, impact and possible management.

IMPORTANCE OF FUNGAL PATHOGENS IN AQUACULTURE

Fish represent a key ecosystem service for fisheries and aquaculture across the world (Zhao et al., 2014). The annual harvest is about 42 million tonnes (marine and freshwater) and the sector employs 33.1 million people, highlighting the tremendous social cost of fisheries (Gozlan and Britton, 2014). The livelihoods of 60 million people in the developing world are dependent on river fisheries and millions more rely on them for food (Dugan and Allison, 2010). However, disease in aquaculture represents the most significant economic losses and in particular fungal infections, which in terms of economic impact are second only to bacterial diseases (Neish and Hughes, 1980; Noga, 1993; Bruno et al., 2011; Ramaiah, 2006; Van West, 2006; Gonçalves and Gagnon, 2011). For example it has been reported in Japan some annual losses of 50% in the production of coho salmon *Oncorhynchus kisutch* and elvers of eel *Anguilla Anguilla* due to outbreaks of *S. parasitica* (Hatai and Hoshai, 1994; Scarfe, 2003).

In the last decades, the aquaculture sector has seen a change in the fish production with a trend toward intensification with the use of recirculating systems (Larkin and Sylvia, 1999). The underpinning drive was a reduction of environmental footprint, a better control of the rearing environment and increased biosecurity. Nonetheless, this improved control of rearing conditions, has lead the industry to also increase the stocking densities of target fish. Thus, it has resulted in an increase of disease outbreaks, with faster transmission and increased mortalities (Bondad-Reantaso et al., 2005; Whittington and Chong, 2007; Peeler et al., 2010; Gonçalves and Gagnon, 2011).

One of the key risks associated with this new aquaculture environment is the stress caused by intensive production. Some fungal pathogens such as *Saproglegnia* for example are more prevalent and virulent in host (salmonids in particular) that are raised under stressful conditions (Willoughby and Pickering, 1977; Willoughby, 1978; Jeney and Jeney, 1995). However, other significant pathogen risks in aquaculture arise from the large and frequent movement of young stages due to either a lack of or insufficient national production, or due to fish species for which the life cycle has not yet been mastered at a commercial level, or even contaminated sources of water supplies. Of course this is not specific to fungal or fungal-like pathogens but their generalist and opportunistic nature associated with a wide environmental tolerance are risk factors that may lead to significant loss of production (Harrell et al., 1986; Paley et al., 2012).

In addition, mycoses spread in fishes are often seen as a secondary phenomenon. However, due to their virulence, their current emergence in wild fish populations and also the risk of spill back from aquaculture facilities to the wild, routine pathological examination should include (in addition to bacteriological ones) mycological examination (see Rehulka, 1991 for details). Dominant fungal pathogens reported in aquaculture are oomycetes including the genera *Achlya*, *Aphanomyces* and *Saprolegnia* (Willoughby and Pickering, 1977; Blazer and

Wolke, 1979; Noga, 1993). *A. invadans* for example can cause epizootic ulcerative syndrome in over a hundred of mostly freshwater fish (e.g., Vishwanath et al., 1998; Blazer et al., 2013; Nsonga et al., 2013) but also in some brackish fish species (Catap and Munday, 2002; Sosa et al., 2007b). In aquaculture conditions, the most appropriate control is through eradication of the stock, quarantine of new stocks and good husbandry (Scarfe, 2003; Whittington and Chong, 2007) and as such represent a significant cost to the trade (Forneris et al., 2003).

There are no treatments that are specific to fungal and fungal-like pathogens but existing ones such as the use of hydrogen peroxide or formalin (Arndt et al., 2001), malachite green (Van West, 2006), sodium chloride (Schreier et al., 1996) and bronopol (Shinn et al., 2012) all present some significant issues related to either human or fish health or to efficacy of the treatments (Carana et al., 2012). Malachite green was banned by the US and EU in early nineties and since then formalin has probably been the most effective control measure but there is a strong possibility that this will soon also be banned from use. Other treatment such as bronopol and other agents are not as effective. Other treatments such as the use of ozone in recirculating systems have to be specifically adapted for fugal pathogens. For example, studies have shown that ozone treatments for *Saprolegnia* are effective with doze from about 0.01 to 0.2 mg.L^{-1} (Gonçalves and Gagnon, 2011) but present a cost of through reduction in hatching rates (e.g., 42.6–49.1%). New treatments based on plant extract have shown some promising paths but further evaluations need to be performed before its use by the industry (Carana et al., 2012).

ECOLOGICAL IMPACT ON WILD POPULATIONS

The emergence of infectious diseases caused by fungal and fungal-like microbes continues to negatively impact wild fish populations, leading in some cases to local and pan-continental extinctions (Gozlan et al., 2005, 2010; Rowley et al., 2013). Thus, understanding of the true ecological cost of fungal and fungal-like microbes is pivotal to improve our conservation practices of fish populations, especially freshwater species, as declines in populations, species distributions and species diversity continue to occur at alarming rates (Myers, 1993; Singh, 2002; Romansic et al., 2009).

Fungal and fungal-like microbes that cause disease emergence in wild fish (**Table 2**), crayfish, amphibians and other aqutic taxa include *Saprolegnia, Batrachochytrium, Ichthyophonous, Aphanomyces, Achyla* and *Sphaerothecum* (Bruno et al., 2011; Swei et al., 2011). *Saprolegnia* and *Sphaerothecum* spp. are impacting wild salmon populations around the world (Willoughby et al., 1983; Van West, 2006; Andreou et al., 2009), prevailing in 32% of adult late-fall-run chinook salmon returning to Battle Creek on the Upper Sacramento River (Arkush et al., 1998). *Aphanomyces* spp. are also responsible for causing EIDs, for example, Epizootic Ulcerative Syndrome (EUS) commonly known as red spot in over a hundred freshwater and estuarine fish species worldwide (Chinabut et al., 1995; Lilley et al., 1997; Boys et al., 2012). EUS has been recognized in Australia (Huchzermeyer and Van der Waal, 2012) and the Philippines (Callinan et al., 1995) since 1972 and 1995 respectively, however in 2006 this fungal pathogen was sighted in the Zambezi River System (ZRS), Africa, the pathogen had travelled further along the ZRS inhabiting several new ecosystems (Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013). Additionally, *Aphanomcyes* spp. low host specificity increases its prevalence among a range of species, increasing disease outbreaks in the ZRS, which is home to approximately eighty species and thus becoming a great concern in disease control (Huchzermeyer and Van der Waal, 2012).

However, compared to farmed fish populations, monitoring EIDs in wild populations can prove difficult as fish are constantly moving long distances beneath turbid waters, which means they can go undetected and underreported distorting our understanding of the effects on these populations (Gozlan, 2012). Globally, EIDs have caused high mortalities in farmed populations (Torto-Alalibo et al., 2005; Phillips et al., 2008; Van Den Berg et al., 2013). This is important information for wild populations because there are several instances where transmission of fungal and fungal-like microbes can occur between the two environments. For example, farmed fisheries often drain into rivers (Andreou et al., 2012) allowing the transfer of microbes and other organisms (Krkosek et al., 2005; Hilborn, 2006). *Pseudorasbora parva* (topmouth gudgeon), widely known by its aquaculture and ornamental fish trade, is a healthy carrier of *S. destruens* (Gozlan et al., 2005). Originally and unintentionally imported from China, the topmouth gudgeon's propitious nature has allowed it to become a profound invader in wild environments, invading thirty five new countries over the past 40 years, where for example *S. destruens* was identified in river systems of the Netherlands and the UK, posing great threats to native fish populations (Gozlan et al., 2010; Spikmans et al., 2013).

Previous studies have shown that susceptibility of farmed fish to fungal or fungal-like microbes depends on several factors including rapid drops in ambient temperatures (Bly et al., 1993; Lategan et al., 2004), low water levels, failure to remove dead fish or eggs, primary infection by other organisms, (Piper et al., 1982; Plumb, 1984) and pollution (Wu et al., 2010), all of which can reduce ecosystem function (Chapin et al., 2000; Cowx and Gerdeaux, 2004; Peeler et al., 2010) and lead to an increase in EIDs (Woolhouse and Gowtage-Sequeria, 2005). Thus, we could expect that such environmental drivers at play in the wild would potentially have also a direct impact on the emergence of fungal pathogens. In particular, the recent paper by Vörösmarty et al. (2010) shows that 65% of rivers worldwide regarding thermal and water level disturbances are under moderate-high threat, in particular Asia and North America. However, Vörösmarty et al. (2010) goes on further to highlight the fact that there is a lack of knowledge and investment being directed to biodiversity conservation, with an increase in EIDs, species distinctions, human population, climate change and habitat destruction (Vörösmarty et al., 2010; Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013). It will be important to monitor these river systems and to reduce these pressures, consequently allowing the populations, species distributions and diversity of fish to remain sustainable for the future.

PERSPECTIVES AND CONCLUSIONS

Since, the initial discovery of the fungal chytrid pathogen 20 years ago (Berger et al., 1998), several studies have reported its

Table 2 | Example of fungal infections in wild fish population.

Host (Family)	Pathogen	Location	Prevalence	Mortality	References
SALMONIDS					
Oncorhynchus tshawytscha (Chinook Salmon)	*Saprolegnia parasitica*	Columbian & Snake Rivers,	–	22%	Neitzel et al., 2004
Oncorhynchus mykiss (Rainbow Trout)		United States of America.			
Salmo salar (Atlantic Salmon)	*Saprolegnia diclina*	River North Esk, Scotland.	30%	–	Roberts et al., 1972
Salmo trutta (Sea Trout)					
Oncorhynchus tshawytscha	*Sphaerothecum destruens*	Sacramento River, United States of America.	32%	–	Arkush et al., 1998
CLUPEDIDS					
Clupea harengus (Bony Herring)	*Ichthyophonus hoferi*	Skagerrak-Kattegat Area, Sweden.	1.1%	8.9%	Rahimian and Thulin, 1996
CHARACIDS					
Astyanax eigenmanniorum *Astyanax fasciatus*	*Saprolegnia parasitica*	Central Argentina.	95%	–	Mancini et al., 2008
CICHLIDS					
Sargochromis giardia (Pink Bream) *Brycinus lateralis* (Stripped Robber)	*Aphanomyces invadans* (EUS)	Zambezi River System, Africa.	3–37.5%	–	Huchzermeyer and Van der Waal, 2012; Nsonga et al., 2013
CYPRINIDS					
Pseudorasbora parva (Topmouth Gudgeon)	*Sphaerothecum destruens*	Meuse River, Netherlands.	67–74%	–	Spikmans et al., 2013
Leucaspius delineates (Belica)	*Sphaerothecum destruens*	Stoneham Lakes system, United Kingdom.	5%	–	Andreou et al., 2011
PERCIDIDS					
Leiopotherapon unicolor (Spangled Perch) *Macquaria ambigua* (Golden Perch)	*Aphanomyces invadans* (EUS)	Murray-Darling River System, Australia.	10% (2008) 29% (2010)	–	Boys et al., 2012

significant impact on amphibians along with major population declines worldwide (Skerratt et al., 2007). What is interesting with this particular pathogen is the relatively good epidemiological data, which have allowed the progression of the disease to be tracked on a global scale and in many wild amphibian populations.

However, there is currently not enough epidemiological data related to fungal pathogens of fish. In light of the recent emergence of *S. destruens*, which poses a threat to European fish diversity (Gozlan et al., 2005; Andreou et al., 2012), it is likely that patterns of ecological impacts similar to those found in chytrid parasites of amphibians, are at play in freshwater fish populations (Gozlan, 2012). For example, as the great majority of *S. destruens* cases are driven by the invasion of a healthy fish carrier, it is expected, as shown by Spikmans et al. (2013), that additional monitoring of invaded wild fish communities would show the presence of this fungal-like infectious pathogen. In fact, there is not enough fungal pathogen data mostly for fish from wild populations. It is interesting to note that the dominant reporting of fish fungal and fungal-like pathogens has come from the aquaculture sector with very limited reports on fungal emergence in wild fish populations (see **Table 2**). The key reason is likely to be a combination of a lack of external pathological specificity of infected hosts and the chronic nature of some of the diseases caused by these fungal and fungal-like pathogens, which, in contrast to viral pathogens, spread over longer periods of time.

Research should be modeled after the amphibian chytrid research structure with a lot more systematic tracking of these pathogens. For example, the recent paper by McMahon et al. (2013) clearly indicates that even for the chytrid, which has been well studied in the wild, new potential non-amphibian hosts could contribute further to its dispersal, prevalence and virulence. Similarly, it would thus be pertinent to determine if fungal and fungal-like pathogens of fish that have a high generalist index could include non-fish hosts and thus contribute to a wider dissemination of some fungal related diseases beyond the immediate local fish communities. Additional monitoring of wild fishes is also needed. Identification and surveys of environmental drivers of fungal pathogens would improve understanding of the ecological risks of disease emergence in aquatic communities (Copp et al., 2009, 2010). This should also be the concern of the aquaculture sector, as strong pathways exist between wild and farmed fish with truly biosecure fish farms being the exception. The pan-extinction of sunbleak *Leucaspius delineatus* populations in Europe in less than 40 years should be a reminder of the risk associated with an un-controlled epizooty of fungal pathogens

(see Gozlan et al., 2005, 2010). Environmental surveys to identify ecological drivers of fungal pathogens in fishes are also key in characterizing the underpinning drivers of fungal and fungal-like pathogen emergence. For example, fish fungal pathogen emergence such as the EUS in Africa could well be linked to current environmental changes occurring in African rivers (Vörösmarty et al., 2010). Thus, in light of the importance of freshwater fish for millions of people around the world, particularly in developing countries, in addition to biodiversity conservation perspectives, pathologists should make a concerted effort to increase their monitoring of fungal pathogens in wild fish populations. Currently, PCR is the method that should be used for monitoring. Along with an increasing reduction in PCR associated cost, their sensitivity and specificity should facilitate such regular monitoring of the wild fish compartment.

In conclusion, our review of fungal and fungal-like pathogens of fish has highlighted current knowledge gaps that need to be rapidly filled if future epizootics are to be prevented. It has also indicated that epidemiological elements arising from other non-fish specific fungal pathogens could be used to refine our true understanding of current and future ecological impacts of these types of pathogens on global fish diversity. For example, existing experimental data arising from fungal pathogen challenges of fish should be used to develop SEIR models (i.e., susceptible-exposed-infectious-recovered) specific to fungal pathogens and fish hosts. This would allow a simulation of the true extent of the ecological risk and provide elements for a better environmental monitoring and understanding of these types of pathogens.

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Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries

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Indigenous microbiota play a critical role in the lives of their vertebrate hosts. In human and mouse models it is increasingly clear that innate and adaptive immunity develop in close concert with the commensal microbiome. Furthermore, several aspects of digestion and nutrient metabolism are governed by intestinal microbiota. Research on teleosts has responded relatively slowly to the introduction of massively parallel sequencing procedures in microbiomics. Nonetheless, progress has been made in biotic and gnotobiotic zebrafish models, defining a core microbiome and describing its role in development. However, microbiome research in other teleost species, especially those important from an aquaculture perspective, has been relatively slow. In this review, we examine progress in teleost microbiome research to date. We discuss teleost microbiomes in health and disease, microbiome ontogeny, prospects for successful microbiome manipulation (especially in an aquaculture setting) and attempt to identify important future research themes. We predict an explosion in research in this sector in line with the increasing global demand for fish protein, and the need to find sustainable approaches to improve aquaculture yield. The reduced cost and increasing ease of next generation sequencing technologies provides the technological backing, and the next 10 years will be an exciting time for teleost microbiome research.

Keywords: fish, microbiota, probiotics, aquaculture, fisheries

INTRODUCTION

The bacteria that colonize the internal and external epidermal surfaces of metazoans are thought to outnumber their host cells by at least 10 to 1 (Human Microbiome Project, 2012). Adult humans contain over a kilogram of such organisms (Ley et al., 2008; Human Microbiome Project, 2012; Karlsson et al., 2013). The emergence and evolution of metazoan organisms has undoubtedly involved close partnership with bacterial life. As such, the relationship that exists between vertebrates and their bacterial colonists dates back hundreds of millions of years (Ley et al., 2008). The microbial metagenome dwarfs that of their hosts (Qin et al., 2010). Numerous metabolic processes vital for host fitness and survival may be assigned to, or facilitated by, their microbial community.

Definition of the services provided by a host microbiome depends on our ability to establish its composition and functional capacity. Furthermore, functional stability in space and time may provide clues to recruitment and host fitness constraints on community structure (Costello et al., 2009; Turnbaugh et al., 2009a). Next-generation sequencing techniques, including amplicon and shot-gun approaches, and associated bioinformatic tools have revolutionized our ability to count and classify commensal bacteria. Concurrently, DNA database development for reliable classification of taxonomy (e.g., GreenGenes, Silva), and functionality (e.g., UniProt, Swiss-prot) has facilitated data interpretation. Large-scale multi-partner projects, particularly the Human Microbiome Project (2012), have driven much of the tool development in this area and are also responsible for the instigation of standard operating procedures to facilitate comparisons between samples, centers, and studies. As such, sophisticated hypotheses across large and dispersed cohorts of individuals can be addressed including the impacts of lifestyle, (e.g., Turnbaugh et al., 2006), disease (Morgan et al., 2012), and antibiotic treatment (Perez-Cobas et al., 2013). Studies frequently document perturbations in meta-community structure that accompany these phenomena as well as perturbations that may have a predictive value for certain metabolic diseases (collectively called dysbiosis) (Karlsson et al., 2013). More important still is to establish a causal link between dysbiosis (imbalance in the microbiome) and pathology. In proving causality, "forward microbiomics" are highly attractive (introducing artificial or transplanting microbiomes into naïve hosts). Humanized germ free (gnotobiotic) mouse models, transplanted with human fecal microbiomes, have corroborated dietary microbiome shifts observed in the clinic (Turnbaugh et al., 2009b). Furthermore, transplantation of "obese" human microbiomes into germ-free animals can modulate mouse metabolism toward adiposity and increased body mass (Ridaura et al., 2013).

Teleost microbiome research lags well behind that in humans and mouse models. Nonetheless, thanks in part to the efforts of Rawls and collaborators, the nature of the Zebra fish gut microbiome was established relatively early in the meta-sequencing goldrush. Their work revealed fascinating reciprocal differences between mammalian and teleost microbiota, as well as the first gnotobiotic teleost model (Rawls et al., 2006). Later studies revealed a "core microbiome" among this species, dominated by γ-Proteobacteria and enriched with a diverse assemblage of Fusobacteria species (Roeselers et al., 2011). Importantly, striking similarities were observed between the microbiomes of domesticated and wild individuals, implying a role for host selection on microbiota, and to an extent validating the conclusions of previous laboratory studies. As well as *D. reria*, several other teleost species have had their microbiota scrutinized via either culture dependent or independent techniques. Studies conducted to date, the tools used and species examined, are summarized in **Table 1**, and a broad overview of their rather incomplete findings in **Figure 1**. Unsurprisingly the focus has been aquaculture species, although some wild individuals have also been studied. Overall there has been important progress in recent years, albeit uncoordinated and sporadic.

In humans, our burgeoning understanding of our "second genome" is driving research into disease, nutrition, lifestyle, as well as immunity and development, (e.g., Furusawa et al., 2013). The applications of an improved understanding in terms of biomarkers, modulation of dysbiotic microbiomes with pre- and pro- biotics, treatment of infectious disease, as well as the generation of totally artificial microbiomes, are considerable. In teleosts, and especially in aquaculture, these applications are equally, if not more, important. Multiple phenomena could be potentially addressed through microbiome manipulation: nutrient digestion, synthesis, absorption, pathogen resistance, growth, sexual maturation, morphogenesis, survivorship in stocked fish, to name a few. In this review we asses the status-quo of teleost microbiome research with special reference to research applications in aquaculture.

TELEOST MICROBIOMES IN HEALTH AND DISEASE

TELEOST MICROBIOMES AS BIOMARKERS FOR STRESS

Aquaculture is a growing industry. Average annual per-capita consumption of fish increased from 12.6 kg in the 1980s to 17.0 kg in 2007, meanwhile wild fish stocks are in steep decline (FAO, 2010). Unfortunately, the growing demand for fish has resulted in an intensification that impacts the welfare of animals in aquaculture systems (Ashley, 2007). Fish welfare in aquaculture may be measured via several physiological and behavioral proxies. These proxies can be usefully combined under the phenomenon of stress. The notion of stress in aquaculture is described by Barton and Iwama (1991) as a normal adaptive physiological response to overcome a negative environmental stimulus or disturbance (Barton and Iwama, 1991). In practice stressful stimuli have multiple sources—handling, sorting, grading, transport and stocking, for example. When such stimuli promote a prolonged stress response, the response may be considered maladaptive as the stress becomes detrimental to fish health.

Microbiome balance is known to be key to maintaining overall health in fish (Gómez and Balcázar, 2008). Stress can influence the microbiome in different ways with repercussions for physiological, hormonal and cellular function. The response of the teleost epidermal mucosa to stress is associated with mucus protein compositional shift (Wendelaar Bonga, 1997; Easy and Ross, 2009; Rakers et al., 2010). The composition of the mucosa in turn shapes their microbial community, and there is evidence that stress impacts microbiome diversity in *Salvelinus fontinalis* (Boutin et al., 2013b). Network analysis of bacterial taxa present in the epidermal mucous of this salmonid during a period of prolonged artificial hypoxic stress revealed interactions between multiple bacterial players in the microbiome. Two taxonomic consortia (co-occuring taxa) emerged (Boutin et al., 2013b). The first consortium, found on unstressed control fish, comprised species from genera *Sphingomonas*, *Methylobacterium*, *Propionibacterium*, and *Thiobacter*, some of which are associated with pro-biotic and/or anti-microbial activity. The second consortium, found on stressed individuals, contained an array of different putative pathogens from the genera *Psychrobacter*, *Steroidobacter*, *Pseudomonas*, *Acinetobacter*, and *Aeromonas.* A conceptual overview of microbiome disruption (or "dysbiosis" as it is often termed) in the epidermal mucous of a teleost is presented in **Figure 2**.

At the most basic level, microbiome homeostasis (stability) is thought to be under the control of constitutively molecules and receptors of the innate immune system (Dixon et al., 2004). Stress is known to impact immunity in several teleost species (Barton and Iwama, 1991; Iger et al., 1995; Espelid et al., 1996). Teleosts share many components of innate immunity with mammals (Magnadottir, 2006). It seems likely that microbiome shifts in response to stress to an extent reflect a shift in host pattern recognition pathways. Thus, indigenous microbiota represent a valuable extension to the standard behavioral and physiological markers of stress. As we will see, stress related imbalance in the microbiome could be a precursor to disease, and thus of crucial practical importance in aquaculture.

TELEOST MICROBIOMES IN COMMUNICABLE DISEASE

The immune system and commensal microbiome are though to form an integrative system of defense from communicable disease (Kitano and Oda, 2006). This system operates on two levels. Firstly, there is now good evidence that the presence of commensal bacteria facilitates the development of the vertebrate adaptive immune system (Rakoff-Nahoum et al., 2004; Kelly et al., 2005; Mazmanian and Kasper, 2006; O'Mahony et al., 2008; Hooper et al., 2012). Furthermore, the commensal microbiome inhibits colonization by pathogenic bacteria either passively, via competitive exclusion, or actively, via toxic secondary metabolites. This effect is termed "colonization resistance" (Wells et al., 1988; Balcazar et al., 2006; Stecher and Hardt, 2008). Any disturbance to the commensal microbiome, which results in dysbiosis, can thus enhance susceptibility to disease (**Figure 2**).

Bacterial pathogens that infect teleosts are found across multiple genera including members of Vibrio, Streptococcus, Aeromonas, Flavobacterium, Photobacterium, Pasteurella, Tenacibacterium, Pseudomonas, Lactococcus, Edwarsiella,

Table 1 | Studies evaluating the diversity of teleost-associated microbial communities.

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Di Maiuta et al., 2013	Panaque sp. (catfish)	A	Faeces samples externally	143,670	16S/454 pyroseqeuncing	Fusobacteria, Cyanobacteria, Beta-proteobacteria, Flavobacteria, Clostridia + other minor groups	Putative cellulolytic bacteria identified Aeromonas sp., Flavobacterium sp., Bacteroides sp., Clostridium sp., and Pseudomonas sp.
Desai et al., 2012	*Oncorhynchus mykiss*	A	Intestinal contents	99,568	16S/454 pyroseqeuncing + DGGE	Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes	NA
Ye et al., 2014	*Dorosoma cepedianum*	W	Intestinal mucosa and contents	400,000+	16S/454 pyroseqeuncing	Cyanobacteria/Cholorplast, Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes, Fusobatceria, Planctomycetes, Chloroflexi, Crenarchaeota	Significant differences between foregut and hindgut microbiota, but not between species
Ye et al., 2014	*Hypophthalmichthys molitrix*	W	Intestinal mucosa and contents	400,000+	16S/454 pyroseqeuncing	Cyanobacteria/Chloroplast, Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes, Fusobatceria, Planctomycetes, Chloroflexi, Crenarchaeota	Significant differences between foregut and hindgut microbiota, but not between species
Geraylou et al., 2013	*Acipenser baerii*	A	Hindgut contents	29,318	16S/454 pyroseqeuncing	Fusobacteria/Firmicutes, Chlamydiae, Bacteriodetes, Actinobacteria	Arabinoxylan oligosaccharide prebuiotics modulate hindgut microbiome composition
Star et al., 2013	*Gadus morhua*	W	Intestinal contents	280,447	16S/454 pyroseqeuncing	Proteobacteria (mostly Vibrionacae), Bacteriodetes, Firmicutes, other minor groups	Large inter-indivudual differences in community composition for fish captured at the same site
Li et al., 2013	*Cyprinus carpio* (transgenic)	A	Intestinal mucosa and contents	621,110	16S/454 pyroseqeuncing + DGGE	Proteobacteria, Fusobacteria, Bacteroidetes, Firmicutes	Differential abundance of bacterial phyla between fast growing transgenic and wild type. Firmicutes: Bacteriodetes ratio differences between transgenic and wild type
Semova et al., 2012	*Dario rerio*	A	Hindgut	10,000+ (data not shown)	16S/454 Pyrosequencing	Firmicutes, Proteobatceria, Bacteriodetes + minor phyla	Microfolora enhance fatty acid uptake in the zebrafish intestine
Wu et al., 2012b	*Ctenopharyngodon idellus*	A	Intestinal mucosa	93,991	16S/454 Pyrosequencing	Firmicutes, Baceriodes, Proteobatceria, Spirochaetes	Cellulose digesting genera present—Anoxybacillus, Leuconostoc, Clostridium, Actinomyces, Citrobacter

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Wu et al., 2012b	*Ctenopharyngodon idellus*	A	Intestinal contents	93,991	16S/454 Pyrosequencing	Firmicutes, Cyanobacteria, Proteobacteria, Bacteriodetes	Cellulose digesting genera present—Anoxybacillus, Leuconostoc, Clostridium, Actinomyces, Citrobacter
Roeselers et al., 2011	*Dario rerio*	A/Wild	Intestinal mucosa and contents	22,980	16S/454 Pyrosequencing, Sanger sequence, TRFLP profiling	Proteobacteria, Fusobacteria, Firmicutes, Actinobateria	Core microbiome: γ-Proteobacteria, β-Proteobacteria, Fusobacteria, Bacilli, Flavobacteria, Actinobacteria classes, Aeromonas, Shewanella
Martin-Antonio et al., 2007	*Solea senegalensis*	A	Intestinal mucosa and contents	176	16S/Culture + Sanger Sequencing	alpha-proteobacteria, gamma-proteobacteria, firmicutes	Temperature and diet both influence microbiota present
Sun et al., 2009	*Epinephelus coioides*	A	Intestinal contents	17	16S/Culture + Sanger Sequencing	Beta -proteobacteria, Gamma-proteobacteria, Firmicutes	Species unequally dispersed beween fast and slow growing phenotypes (e.g., *Bacillus pumilis* super-abundant in fast growers)
Huber et al., 2004	*Oncorhynchus mykiss*	A	Intestinal contents	146	16S/Culture + Sanger Sequencing	Beta -proteobacteria, Gamma-proteobacteria	DAPI staining and FISH analysis demoastrate large number of unculturable bacterial species present
Skrodenyte-Arbaciauskiene et al., 2008	*Salmo salar* (juvenile, freshwater)	W	Intestinal contents	52	16S/Culture + Sanger Sequencing	Gamma-proteobacteria, firmicutes	Principal differences were present between *S. trutta* and *S. salmo* were at bacterial species level
Skrodenyte-Arbaciauskiene et al., 2008	*Salmo trutta* (juvenile, freshwater)	W	Intestinal contents	47	16S/Culture + Sanger Sequencing	Gamma-proteobacteria	Principal differences were present between *S. trutta* and *S. salmo* were at bacterial species level
Skrodenyte-Arbaciauskiene et al., 2006	*Salmo trutta* fario	W	Intestinal contents	100	16S/Culture + Sanger Sequencing	Gamma-proteobacteria	Multiple differences at family and species level between populations isolated from two different river systems
Wu et al., 2012a	*Pelteobagrus fulvidraco*	A	Midgut contents, midgut mucus	74	16S/Culture + Sanger Sequencing	Firmicutes, Proteobacteria, Bacteriodetes, Fusobacteria	Different bacterial genera between gut contents and mucosa. Stomach contents conatained Chloroflexi, while mucous Actinobacteria
Cantas et al., 2011	*Salmo salar* (juvenile)	A	Intestinal mucosa and contents	18	16S/Culture + Sanger Sequencing	Gamma-proteobacteria, firmicutes, actinobacteria	Differences between dilpoid and triploid individuals non-significant

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Valdenegro-Vega et al., 2013	*Thunnus maccoyii*	Ranched	Gills, Spleen, Kidneys	24	16S/Culture + Sanger Sequencing	(no order) *Vibrio* and *Photobacterium* sp. predominate	–
Cantas et al., 2012	*Dario rerio*	A	Intestinal contents	13	16S/Culture + Sanger Sequencing	Gamma-proteobacteria, beta-proteobatceria, alpha-proteobatceria, firmicutes	–
Tetlock et al., 2012	*Petromyzon marinus*	A	Intestinal contents	682	16S/DGGE + Sanger sequencing	Proteobacteria	Dominated by Aeromonas species
Shiina et al., 2006	*Takifugu niphobles*	W	Intestinal contents	24	16S/DGGE + Sanger sequencing	Firmicutes, Gamma-proteobacteria, Spirochaetes	Cultivable species restricted in greater part to Vibrio species
Tetlock et al., 2012	*Petromyzon marinus*	A	Intestinal mucosa and contents	682	16S/DGGE + Sanger sequencing	Proteobacteria, Bacteriodetes, Tenericutes, + minor phyla	Hugely diverse environment, multiple genera and species
He et al., 2010	*Oreochromis* sp.	A	Intestinal contents	19	16S/DGGE + Sanger sequencing	Cyanobacteria. Proteobacteria, Firmicutes, Actinobacteria, Fusobacterium	Significant influence on antibiotics on gut microbiota
Silva et al., 2011	*Carassius auratus*	A	Intestinal mucosa and contents	60	16S/DGGE + Culture + Sanger Sequencing	Gamma-proteobacteria, Firmicutes	Dominated by Aeromonas species
Svanevik and Lunestad, 2011	*Scomber scombrus*	W	Gills/skin/inestine contents	99	16S/DGGE + Culture + Sanger Sequencing	Gamma-proteobacteria, Firmicutes	Vibrio, Pscrobatcer immobilis, Oceanisphaera and some Shewanella species only present in the gut (samples direct from purse seine onlu included)
Kühlwein et al., 2013	*Cyprinus carpio* L.	A	Intestinal contents	27	16S/DGGE + Culture + Sanger Sequencing	(no order) Proteobacteria, Firmicutes, Fusobacteria	Dietary β-(1,3)(1,6)-D-glucan supplementation impacts gut microbiota
Kim et al., 2007	*Oncorhynchus mykiss*	A	Intestinal mucosa and contents	199	16S/DGGE + Culture + Sanger Sequencing	Proteobacteria, Fusobacteria	Differences between intestinal mucosa and contents. e.g., Gut contents—Enterobacter, Bacteroides, Flavobacteria, Pasteurellacae. Mucosa =- Enterobacter, Aeromonadacae, Pseudomonadacae, Mycoplasmatacae

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Silva et al., 2011	*Sparus aurata*	A	Intestinal mucosa and contents	160	16S/DGGE + Culture + Sanger Sequencing	Gamma-proteobacteria, Bacteroidetes, Firmicutes	Dominated by Photobacterium sp.
Navarrete et al., 2012	*Oncorhynchus kisutch* (juvenile)	A	Eggs + Juvenile intestinal contents	14	16S/DGGE + Sanger sequencing	Egg—Bacteriodetes (flavobacteria), Beta-proteobacteria; Juvemiles—Gamma-proteobatceria, firmicutes	–
Merrifield et al., 2013	*Dario rerio*	A	Hindgut	8	16S/DGGE + Sanger sequencing	(no order) Fusobacteria, Gammaproteobacteria	Nanoparticles included in diet disrupt communty structure
Ni et al., 2012	*Ctenopharyngodon idellus*	A/W	Intestinal mucosa and contents	75	16S/DGGE + Sanger sequencing	(no order) Cetobacterium. Aeromonas, Plesiomonas, Sporacetigenium, Enterobacter	–
Tapia-Paniagua et al., 2010	*Solea senegalensis*	A	Intestinal contents	7	16S/DGGE + Sanger sequencing	Gamma-proteobacteria	Dominated by *Vibrio* species, enhanced by prebiotics
Zhou et al., 2012	*Gadus morhua*	A	Intestinal mucosa and contents	34	16S/DGGE + Sanger sequencing	Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes, Deinococci	Genera associated with chitin-rich diet: Escherichia, Erwinia, Thermus
Geraylou et al., 2012	*Acipenser baerii*	A	Hindgut	36	16S/DGGE + Sanger Sequencing	Proteobacteria, Firmicutes, Fusobacteria	Comparison of different diets on hind gut fermentation
Liu et al., 2012	*Carassius auratus*	A	Intestinal contents	ND	16S/DGGE + Sanger Sequencing	Actinobacteria, Firmicutes, Proteobacteria	Antibiotic treatment disrupts microbiota of healthy fish more significantly than those with disease.
Li et al., 2012	*Ctenopharyngodon idellus*	A	Intestinal mucosa and contents	41	16S/DGGE + Sanger Sequencing	(no order) alpha, beta and gamma-proteobacteria, Actinobacteria	–
Li et al., 2012	*Hypophthalmichthys molitrix*	A	Intestinal mucosa and contents	41	16S/DGGE + Sanger Sequencing	(no order) Actinobacteria, Firmicutes, alpha and gamma-proteobacteria	–
Li et al., 2012	*Hypophthalmichthys nobilis*	A	Intestinal mucosa and contents	41	16S/DGGE + Sanger Sequencing	(no order) alpha, beta and gamma-proteobacteria, Actinobacteria	–

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Li et al., 2012	*Megalobrama amblycephala*	A	Intestinal mucosa and contents	41	16S/DGGE + Sanger Sequencing	Beta and Gamma-proteobacteria	–
Navarrete et al., 2010	*Salmo salar*	A (marine)	Intestinal mucosa and contents	700	16S/RFLP + Sanger sequencing	Gamma-proteobacteria, Firmicutes, Bacterioidetes	Pseudomonas, Acinetobacter, Flavobacterium, Psychrobacter, Brevundimonas, Caulobacter, Mycoplana, Aeromonas, Haemophilus, Aeromonas salmonicida, Bacillus, Micrococcus/Kocuria. Reduction in diversity among tetracyclin treated indiividuals
Moran et al., 2005	*Kyphosus sydneyanus*	W	Intestinal contents	12	16S/T-RFLP, Sanger Sequencing	(no order) *Closteridium* species	Putative inviolvement in short chain fatty acid metabolism
Smriga et al., 2010	*Acanthurus nigricans*	W	Intestinal contents	48	16S/TA cloning, Sanger sequencing	Bacteriodetes, Firmicutes Proteobacteria (Vibrionacae ijn minority), Bacteriodete, Spirochaetes	–
Smriga et al., 2010	*Chlorurus sordidus*	W	Intestinal contents	44	16S/TA cloning, Sanger sequencing	Proteobacteria (mostly Vibrionacae), Bacteriodetes + other minor groups	–
Ward et al., 2009	*Chaenocephalus aceratus*	W	Intestinal contents	303	16S/TA cloning, Sanger sequencing	Gamma-proteobatceria	Photobacterium
Smriga et al., 2010	*Lutjanus bohar*	W	Intestinal contents	46	16S/TA cloning, Sanger sequencing	Proteobacteria (Vibrionacae), Firmicutes	–
Ward et al., 2009	*Notothenia coriiceps*	W	Intestinal contents	194	16S/TA cloning, Sanger sequencing	Gamma-proteobatceria	Photobacterium/Vibrio
Green et al., 2013	*Salmo salar*	A (marine)	Intestinal contents	30	16S/TA cloning, Sanger sequencing	(no order) Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Verrucomicrobi	Addition of soyabean derived protein resulted in dysbiotic changes in intestinal microbiota and presence of genera not normally associated with the marine environment

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Larsen et al., 2013	*Cynoscion arenarius,*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Bacteriodetes	Fish species, capture locality and capture date all influence skin microbiota
Larsen et al., 2013	*Cynoscion nebulosus*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Bacteriodetes	Fish species, capture locality and capture date all influence skin microbiota
Larsen et al., 2013	*Lagodon rhomboides*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Actinobacteria	Fish species, capture locality and capture date all influence skin microbiota
Larsen et al., 2013	*Lutjanus campechanus*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes, Cyanobacteria	Fish species, capture locality and capture date all influence skin microbiota
Larsen et al., 2013	*Micropogonias undulatus*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteria, Bacteriodetes	Fish species, capture locality and capture date all influence skin microbiota
Larsen et al., 2013	*Mugil cephalus*	W	Skin mucosa	69	16S/TA cloning, Sanger sequencing	Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes, Cyanobacteria	Fish species, capture locality and capture date all influence skin microbiota
Navarrete et al., 2009	*Salmo salar* (juvenile)	A	Intestinal mucosa and contents	80	16S&ITS/TTGE and Sanger sequencing	Proteobacteria	Differences between gut compartments by TGGE
Arias et al., 2013	*Lutjanus campechanus*	W	Anterior Kidney	43	16S/Culture + Sanger Sequencing	Proteobacteria, Firmicutes, Actinobacteria	Firmicultes and Actinobatceria more common on the skin than in the kidney
Ringø et al., 2006	*Gadus morhua*	A	Intestinal mucosa and contents	425	16S/Culture + Sanger Sequencing	(no order) Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria	Dietary differences in microbiota. Bacteriodetes preferentially adherent. Anthrobacter absent from foregut
Arias et al., 2013	*Lutjanus campechanus*	W	Skin mucosa	179	16S/Culture + Sanger Sequencing	Proteobacteria, Firmicutes, Actinobacteria	Firmicultes and Actinobatceria are more common on the skin than in the kidney
Mansfield et al., 2010	*Oncorhynchus mykiss*	A	Ground intestinal tissue	3357	HSP60/Sanger clones libraries	Firmicutes, gamma-proteobacteria, alpha-proteobacteria, actinobacteria	–

(Continued)

Table 1 | Continued

Study	Fish species	Fish origin[a]	Organ	Sequences derived (approx.)	Target/technique	Phyla (order of abundance)	Notable genera/findings
Boutin et al., 2013a	*Salvelinus fontinalis*	A	Skin mucosa	117,260	16S/454 pyroseqeuncing	Proteobacteria (Alpha, Gamma, Beta and Delta), Actinobacteria, Bacteroidetes, Firmicutes, TM7, Chlorobi	Probiotic treatment by an indigenous strain does not disturb the natural microbiota of *Salvelinus fontinalis*
Boutin et al., 2014	*Salvelinus fontinalis*	A	Skin mucosa	87,940	16S/454 pyroseqeuncing	Proteobacteria (Alpha, Gamma), Bacteroidetes	*Salvelinus fontinalis* presents three QTL region linked to the abundance of three commensal genera
Boutin et al., 2013b	*Salvelinus fontinalis*	A	Skin mucosa	678,211	16S/454 pyroseqeuncing	Proteobacteria (Beta, Alpha, Gamma), Actinobacteria, Bacteroidetes	Host' stress influences the skin microbiota. Commensals strains abundance decreases and favors growth of opportunistic pathogens

[a]*Aquaculture, A; Wild, W.*

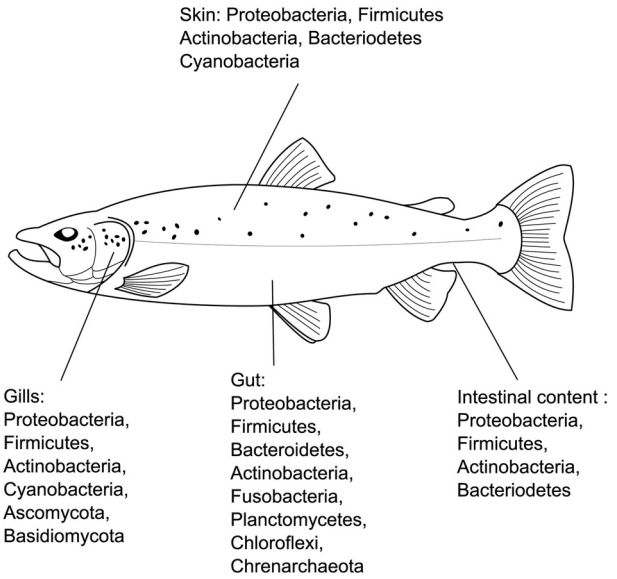

FIGURE 1 | General microbiological findings on fish microbiota. This overview synthesizes the major phyla present in the different organs of fish from different species. Bacterial phyla included are correspond to those which made up >80% of sequences characterized from a given tissue/organ in each study. Only studies that employed direct sequencing (clone libraries/amplico-seq) are included.

Yersinia, Renibacterium, and Mycobacterium (Austin and Austin, 2007). Most of these organisms can survive as well as (in some cases) replicate outside their host in the aquatic milieu. In addition they are almost all opportunistic pathogens (Austin and Austin, 2007). Culture and direct sequencing based surveys of commensal skin and intestinal microbiota suggest bacterial pathogens frequently occur as a minor component of healthy teleost microbiomes but emerge as pathogens under certain circumstances, e.g., (Navarrete et al., 2010; Austin and Austin, 2012; Boutin et al., 2013a,b). Stress, usually of the prolonged, maladaptive type, is perhaps the most commonly attributed as a causal factor in aquaculture disease outbreaks (Snieszko, 1974; Wakabayashi, 1991; Wendelaar Bonga, 1997; Le Moullac et al., 1998; Sudo et al., 2004; Schimel et al., 2007; Freestone et al., 2008; O'Mahony et al., 2009; Thurber et al., 2009; Littman et al., 2010; Boutin et al., 2012; Verbrugghe et al., 2012; Moloney et al., 2013). The link between stress and disease is not limited to bacterial pathogens and teleost aquaculture. White spot syndrome virus (WSSV), for example, a major pathogen in shrimp, is commonly found in healthy populations as a commensal agent, yet the mechanisms for this latency are not clearly understood (Sanchez-Paz, 2010).

As aquaculture intensifies, host population densities have increased to support the kind of virulence shifts associated with pathogenic agents that cause large, horizontally transmitted outbreaks (Pulkkinen et al., 2010). Stress-induced microbiome dysbiosis may be a useful predictor for the emergence of opportunistic disease. However, it is not clear to what extent a healthy microbiome will protect against the more virulent aquaculture pathogens of the future. Furthermore, it remains to be

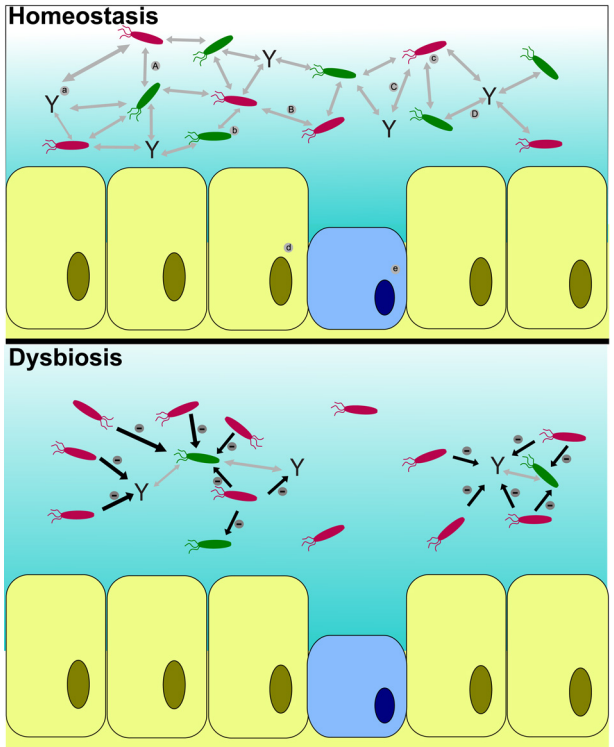

FIGURE 2 | Host microbiota interactions during homeostasis and dysbiosis. The host is able to control the pathogen (c) growth by different process (A,C) involving the immune response (a) and the resident microbiota (b). Furthermore, the immune response recognizes the resident microbiota (D) as non-pathogenic bacteria. Pathogenic bacteria auto-regulate abundance via quorum sensing (B) and can detect environmental signals from host cells [epidermic cells (d) and mucous cells (e)]. During dysbiosis, the pathogenic population, triggered by the stress response of the host (diminution of the immune response, production of mucus and diminution of the abundance of the resident microbiota), overcome the immune response and outcompete the resident microbiota.

seen what role the teleost microbiomes have in defining susceptibility to important ectoparasites in aquaculture (Caligidae, Monogea, etc.), as well as to the secondary bacterial infections they precipitate.

DIET AND THE TELEOST MICROBIOME

Most published work on teleost-associated microbiota focuses on the intestinal microbiome (**Table 1**). Among those experimental studies undertaken, a common line of investigation is the influence of diet on bacterial community composition. Non-marine protein supplementation is a key issue with respect to the aquaculture of predatory marine teleosts. For both *Salmo salar* and *Gadus morhua* supplementation with soya bean derived proteins resulted in significant shifts in intestinal microbiota, including the presence of bacteria atypical to marine environments (Ringø et al., 2006; Green et al., 2013). It is not clear whether these changes may be termed "dysbiotic" as the authors suggest, partly because so little is known about the "natural" state of gut microbiomes in these species. Fortunately recent work has probed the natural diversity of gut microbiota in wild Norweigian cod (*G. morhua*) via Roche 454 pyrosequencing (Star et al., 2013). The study revealed substantial inter-individual variation and suggested a predominance of Vibrionacae (proteobacteria) among the 15+ bacterial orders identified. Meaningful comparison between this dataset and previous, culture based surveys of microbiota in *G. morhua* are essentially impossible, although proteobacteria were been identified using both isolation techniques (Ringø et al., 2006; Zhou et al., 2012). The current technological shift from culture-based isolation and Sanger sequencing to direct PCR amplification and massively parallel sequencing means that meaningful comparisons are thin on the ground. The total number of bacterial sequences derived from *G. morhua* intestinal microbiomes was 459 prior to Star et al. (2013) (Ringø et al., 2006; Zhou et al., 2012; Star et al., 2013). The pyrosequencing Star et al. (2013) undertook increased this tally by 280,447.

Whether or not teleost microbial studies have used the most up-to-date methods for profiling gut bacterial communities, the themes on which they touch are certainly valid, and form a platform for future research. As well investigating the impact of soya protein, researchers have evaluated the impact of dietary chitin on the microbiome (Zhou et al., 2012). Chitin represents a huge, but largely indigestible, potential source of carbohydrates for fish. It is of considerable interest what role indigenous gut microbiota might play in chitin decomposition. Similarly, the presence of cellulolytic microbial species in the intestines of the wood eating catfish has been probed (Di Maiuta et al., 2013). Such studies will benefit from functional characterization of the bacterial metagenetic repertoire, and teleost alimentary tracts promise rich veins for glycide hydrolase bioprospecting, given the huge variety of different dietary niches they exploit.

MICROBIOME MANIPULATION

PROBIOTICS

It is understood that several parameters: genetic, nutritional and environmental; affect the abundance and diversity of gut microbiota in fish (Dimitroglou et al., 2011; Daniels and Hoseinifar, 2014; Ringø et al., 2014). The idea of manipulating gut microbiota of fish developed as a consequence of the fact that potentially beneficial bacterial communities such as lactic acid bacteria naturally constitute only a minor proportion of intestinal microbiota of fish or shellfish (Ringø et al., 2010). It has been suggested that the manipulation of fish gut microbiota will result in elevation of resistance against pathogens, growth enhancement, improved lipid metabolism, stimulation of immune response and better physiological status for the gut (Tellez et al., 2006). Thus, there is increasing interest in strategies for the manipulation of gut microbiota of fish toward beneficial communities (e.g., lactic acid bacteria) (Daniels and Hoseinifar, 2014; Ringø et al., 2014).

A primary approach toward microbiome manipulations is the administration of probiotics, which are defined as live microbial culture added to feed or environment (water) to increase viability (survival) of the host (Gram and Ringø, 2005). This definition is being constantly refined and updated associated with health promoting properties (Irianto and Austin, 2002a) or with other benefits. The latest accepted definition for probiotics for aquatic animals is suggested by Merrifield et al. (2010). According to the authors probiotic for aquaculture is a live, dead or component

of a microbial cell that, when administered via the feed or to the rearing water, benefits the host by improving either disease resistance, health status, growth performance, feed utilization, stress response, which is achieved at least in part via improving the hosts or the environmental microbial balance.

Although the mechanisms by which probiotics exert their beneficial effects on the host are largely unknown, probiotics administration showed promising results on growth performance and health of teleost fish (Gatesoupe et al., 2010). Despite the aforementioned advantages of probiotics, the viability of live bacteria during large-scale production of food (i.e., commercial diets) and during transition through the gastrointestinal tract is not reliable (Ringø et al., 2014).

PREBIOTICS

To resolve issues with probiotics, the prebiotic concept has been suggested and developed (Mahious and Ollevier, 2005). A prebiotic is a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health (Roberfroid, 2007). According to Gibson (2004) a dietary ingredient should meet the following criteria to be classified as a prebiotic, (1) resist gastric acidity, hydrolysis by digestive enzymes and gastrointestinal absorption; (2) be fermented by the intestinal microbiota and; (3) be able to selectively stimulate the growth and activity of beneficial bacteria (Gibson, 2004). To our knowledge the first study on prebiotics in aquaculture was reported by Hanley et al. (1995). Since then the most common prebiotics studied in fish were inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS), *trans*-galacto-oligosaccharides (TOS), Bio-MOS® containing MOS derived from yeast, galacto-oligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), GroBiotic®-A (GBA) (Ringø et al., 2014). Beneficial bacterial members of the gut microbiota use prebiotics as substrate for growth. 454 pyrosequencing has recently confirmed this effect in juvenile Siberian sturgeon (*Acipenser baerii*) fed with an AXOS prebiotic (Geraylou et al., 2012). In this work, significant increases in abundance of several bacterial families, including *Lactobacillaceae*, were observed in individuals with AXOS treatment regimes. Another important product of prebiotic fermentation by gut microbiota is short chain fatty acid (SCFA) (Cummings and Macfarlane, 2002). SCFA are the main energy source for colonic epithelial cells and thus associated with maintenance of the epithelium (Maslowski and Mackay, 2010). Moreover, it has been proposed that SCFA modulates lipid synthesis (Marcil et al., 2002) and has the potential to stimulate the immune system and resistance against pathogens (Maslowski and Mackay, 2010). However, it remains to be seen precisely which microbial taxa play a dominant role in SCFA production in fish.

SYNBIOTICS

A recent concept in regards to the manipulation of gut microbiota are synbiotics. Synbiotics refer to nutritional supplements combining probiotics and necessary nutrients for their survival (Cerezuela et al., 2011). As such, synbiotics aim to simultaneously seed and maintain probiotic strains as the dominant species in the gut after treatment cessation (Rurangwa et al., 2009). Despite recent progress in the field of synbiotics administration in aquaculture, there is limited information available on different aspects of synbiotics' effects on fish (Cerezuela et al., 2011).

PROBIOTICS AND DISEASE

The use of probiotics as biological control agents for disease is fairly well established in aquaculture, in contrast to other areas of animal and human health, where it seems all but absent as an approach (Newaj-Fyzul et al., 2013). Bacterial cultivars from over 30 different genera are have been administered (Newaj-Fyzul et al., 2013). Target disease agents are usually bacterial, and infection with a wide variety of pathogens has been treated in several different teleost species, primarily in aquaculture. *Aeromonas hydrophila* has been successfully used *in vivo* to treat *A. salmonicida* infection in *Oncorhynchus mykiss,* for example (Irianto and Austin, 2002b). Meanwhile *Rhodococcus qingshengii* had been successfully applied to the treatment of *Flavobacterium psychrophilum* infection in *Salevinus fontinalis* (Boutin et al., 2012). There are numerous examples in the literature of such trials (Newaj-Fyzul et al., 2013), however, it is by no means clear by what mode of action these agents operate, especially in the context of the wider microbiome. While some effective probiotics, particularly those administered prior to challenge with the infectious agents, (e.g., De la Banda et al., 2012), may to an extent bolster the "colonization resistance" of the indigenous microbiome, the action of others is less clear still. Longitudinal surveys of the indigenous microbiome during these trials are sparse, and there is clearly significant scope for further research.

HOST GENETICS AND TELEOST MICROBIOMES

The level of influence that host genome exerts on microbiome composition is a matter for debate, even in well-studied organisms like humans (Spor et al., 2011). There is evidence that the quantitive trait loci (QTL) can detect an influence of host genetic variation on fecal microbiome composition in mice (Benson et al., 2010). Those taxa under host genetic control corresponded with species and genera thought to interact with host immunity (Benson et al., 2010). QTL analysis of skin microbiome composition has recently been undertaken in the salmonid *Salvelinus fontinalis* (Boutin et al., 2014) and "common garden experiments" on different *O. mykiss* families have also explored associations with host background (Navarrete et al., 2012). As with mice, in both cases there is some limited evidence for host genetic control. At the inter-species level, there may some level of host-specificity in teleost larvae as well (Li et al., 2012). Given that maternal effects can be largely discounted in fish, the mechanism through which such control is exerted must be innate immunity. Pathogen Recognition Receptors (PRRs)—comprised of Toll-like receptors (TLRs), and their co-receptor CD14, the scavengers receptors, the mannose receptors, the integrins CD11b-c/CD18 and the complement receptors CR1,2,3—form a major component in innate immunity. PRRs are expressed at the surface of the cells to recognize a variety of non-host ligands collectively termed microbe associated molecular patterns (MAMPs) (Medzhitov and

Janeway, 1999). Standing genetic variation among components of the teleost adaptive immune system is increasingly well characterized, (e.g., Dionne et al., 2009; Pavey et al., 2013). While TLRs are present in multiple teleost species (Palti, 2011), there has been no work to date to correlate genetic diversity at these innate immune loci (inter- or intra- species) with commensal microbiome diversity. Experiments in zebrafish highlight the role that TLRs play in modulating intestinal microbiota, whereby alkaline phosphatase is produced via a TLR-4-myD88 controlled pathway to inhibit an inflammatory responses to gut microbiota (Bates et al., 2007). Given that desirable microbiome characteristics from an aquaculture perspective may exist (e.g., disease resistance, nutrient absorption, stress resilience), it is encouraging that a host genetic basis may exist to select for such traits.

MICROBIOME ONTOGENESIS

Intensive aquaculture is hampered by unpredictable mortalities during early life stages that are likely due, at least in part, to negative interactions between fish larvae and some bacterial strains they routinely encounter. In order to control mortalities at early life stages, the aquaculture industry prioritized egg and larvae disinfection protocols. Such guidelines are perhaps counter-productive, given that most of the bacteria routinely isolated from hatcheries are not harmful to larvae (Verner-Jeffreys et al., 2003), and fish microbiota are the first line of defense against pathogens (Boutin et al., 2012).

Early promotion of nutrient metabolism and innate immune response depend upon the bacterial species that colonize the digestive tract. It is therefore of primary importance to understand the mechanisms that orchestrate the early steps of colonization of the gastrointestinal tract of fish, leading the buildup of a stable, diversified and resilient endogenous microbial community. Colonization steps are summarized in **Figure 3**.

Culture-based identification of bacterial species has been the mainstay of studies examining early teleost microbiome development to date, but their finding are nonetheless intriguing. In the aquatic environment, bacteria move easily between habitats and hosts. Thus the first steps of interactions and colonization of fish progeny occur as soon as the eggs are laid. The number of bacteria colonizing salmonid eggs, for example, ranges between 10^3 and 10^6 bacteria g^{-1} (Yoshimizu et al., 1980). The diverse microbiota that eventually develops on the egg surface is expected to reflect the bacterial composition of the water. Interestingly,

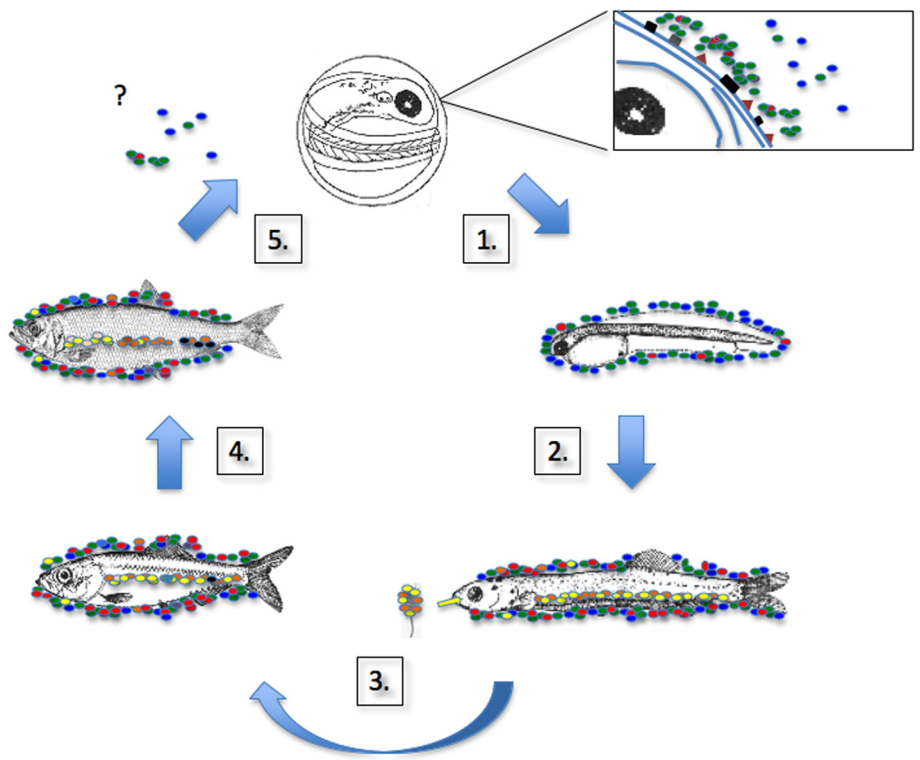

FIGURE 3 | Teleost microbiome during development. Figure shows schematic of the generalized lifecycle of a teleost and accessory indigenous bacteria (different taxa represented by colored elipses). (1) Bacteria colonize the chorion of the egg. Taxonomic differences of bacteria between fish species suggest specific early interactions, perhaps through precursors of innate immunity (symbolized by squares and triangles on the chorion surface). (2) Egg hatches, larval is colonized by environmental bacteria as well as those originally present on the chorion. (3) Early digestive tract colonization occurs when larva commence feeding. Bacterial taxa strongly resemble those associated with food source. (4) Microbiome develops, accumulates diversity and matures. (5) Adult microbiome is diverse assemblage of microbial taxa. Differences exist between surface mucosal and intestinal communities. Intestinal communities also be compartmentalized/specialized to niches within the alimentary tract. Question mark indicates possible vertical transmission of microbiome components to eggs during oviposition.

species-specific differences were observed in terms of bacterial colonization of fish eggs between cod and halibut (Hansen and Olafsen, 1989). Such host specific assemblages on the chorion may result from differential attraction to surface receptors, to those being coded by host genotype. Once eggs hatch, sterile larvae are rapidly colonized by ova debris and microbiota present in the environment (Hansen and Olafsen, 1989). Passage of surface bacteria into the gut is expected to colonize larvae gut as soon they are begin to ingest their liquid medium (Lauzon et al., 2010). Unsurprisingly, the alimentary tract of first-feeding fries is colonized with bacteria associated with food (Blanch et al., 1997; Korsnes et al., 2006; Reid et al., 2009). The process of recruitment of taxa to the developing microbiome clearly has to work with those bacteria present in the immediate environment.

Romero and Navarrete (2006) pioneered the identification of dominant bacterial populations associated with early life stages of salmon coho using a 16S RNA barcoding approach using a DGGE metagenomic (culture-independent) approach (Romero and Navarrete, 2006). They focused on three developmental stages (eggs, first-feeding fry, juvenile) and documented environmental bacterial communities (surrounding water, pelletized feed) in order to determine the putative origin of dominant intestine tract strains. Interestingly, a dominant *Pseudomonas* sp. found in the juvenile gastrointestinal tract was also present on eggs, but not in the water nor in food. This may suggests a vertical transmission of a pioneering strain, which is commonly observed as a dominant genus in gut microbiota of mature fish (Hansen and Olafsen, 1999; Jensen et al., 2004; Navarrete et al., 2010). Overall, DGGE profiles showed pioneering communities harboring very few ribotypes, those encountered important shifts, at least in terms of taxonomic diversity, between eggs, first-feeding fry, and juvenile step. The authors concluded that the early steps of the gut microbiota colonization by bacterial strains do not reflect a stable microbiota, which would be established after the first feeding stages, by recruiting its major components from water and prey epibiota. Such finding corroborates the observation that during the initial stage of gut colonization, microbiota is highly unstable in humans (Palmer et al., 2007; Mariat et al., 2009; Cho and Blaser, 2012) and mice (El Aidy et al., 2012, 2013). Furthermore, the temporal pattern in which gut microbiota evolves is characterized by a remarkable interindividual variation. Over time, microbial groups that typically dominate the adult intestinal microbiota overcome the early-colonizing microbes that are less adapted to the intestinal environment (Palmer et al., 2007; El Aidy et al., 2013).

Because the early stages of fish development are the most sensitive regarding to outbreak caused by opportunistic pathogens, and because fish microbiota are now understood as the very first barrier against opportunistic pathogens, it is of primary importance to identify the factors that control the early steps of colonization of the fish microbiota, in order to maximize the rearing conditions leading to the buildup of a stable, diversified and resilient endogenous microbial community. Gnotobiotic models starting with germ-free larvae provide an excellent tool to disentangle accurately the host microbe interactions (Rawls et al., 2004, 2006; Dierckens et al., 2009; Rekecki et al., 2013; Rendueles et al., 2013). For example zebrafish (*Danio rerio*), a widely used cyprinid fish as a valuable vertebrate developmental model, proved to be convenient for studying gut microbiota ontogenesis, host-microbiota and host-pathogen interactions (Rawls et al., 2004, 2006; Kanther, 2010). Thus, far, more than 20 pathogenic strains have been tested on germ free zebrafish (van der Sar et al., 2004; Lesley and Ramakrishnan, 2008; Kanther, 2010; Kanwal et al., 2013) or colonized with an artificial microbiota (Rawls et al., 2006; Cheesman and Guillemin, 2007; Kanther et al., 2011). Similar experiments were undertaken in non-model fish such as cod (Forberg et al., 2012), sea bass (Rekecki et al., 2013), and halibut (Verner-Jeffreys et al., 2003). In general, the results from most studies involving challenge of wild type or germ-free fish larvae with opportunistic pathogens highlight the protective role of the indigenous bacteria (Kanwal et al., 2013; Rendueles et al., 2013).

CONCLUSIONS

At the time of writing, teleost microbiome research is on the cusp of significant progress. Next generation sequencing is increasable affordable, computationally achievable in small laboratories, and generally accessible to the wider scientific community outside model vertebrates. In this review we have highlighted areas of current interest for teleost microbiome research, namely as biomarkers for stress and disease resistance. Diet is also a major area for microbiome research, especially with regards to new feed sources to mitigate the environmental impact of aquaculture. We discussed current approaches to directly manipulate host microbiomes via pro-, pre- and synbiotics in an attempt to improve fish condition and treat disease, as well as a host genetic basis for microbiome diversity, which could be used to select for desirable microbiome traits in the future. Finally we touched on microbiome ontogenisis in juvenile fish, crucial for the development healthy digestion and immunity.

Whilst the areas of research interest have largely been defined, the technology will shortly shift. Once next generation sequencing is routinely used to characterize teleost microbiomes, it should become significantly easier to make meaningful comparisons between species, studies, research centers and sample sites. In line with approaches defined by the HMP (Human Microbiome Project, 2012), it is extremely important to establish baselines for natural teleost microbiomes before meaningful conclusions can be drawn from the same species in aquaculture. The term "dysbiosis" is currently over-used given that the natural stability (or instability) of any teleost microbiome is not currently known.

The next 5–10 years will be an exiting time for teleost microbiome research. The timing couldn't be better given the parlous state or many wild fish stocks, the increasing global demand for fish protein, and the need to find sustainable approaches to improve aquaculture yield and mitigate its impact on marine and freshwater environments.

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Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity

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Eukaryotic microbes (protists) residing in the vertebrate gut influence host health and disease, but their diversity and distribution in healthy hosts is poorly understood. Protists found in the gut are typically considered parasites, but many are commensal and some are beneficial. Further, the hygiene hypothesis predicts that association with our co-evolved microbial symbionts may be important to overall health. It is therefore imperative that we understand the normal diversity of our eukaryotic gut microbiota to test for such effects and avoid eliminating commensal organisms. We assembled a dataset of healthy individuals from two populations, one with traditional, agrarian lifestyles and a second with modern, westernized lifestyles, and characterized the human eukaryotic microbiota via high-throughput sequencing. To place the human gut microbiota within a broader context our dataset also includes gut samples from diverse mammals and samples from other aquatic and terrestrial environments. We curated the SILVA ribosomal database to reflect current knowledge of eukaryotic taxonomy and employ it as a phylogenetic framework to compare eukaryotic diversity across environment. We show that adults from the non-western population harbor a diverse community of protists, and diversity in the human gut is comparable to that in other mammals. However, the eukaryotic microbiota of the western population appears depauperate. The distribution of symbionts found in mammals reflects both host phylogeny and diet. Eukaryotic microbiota in the gut are less diverse and more patchily distributed than bacteria. More broadly, we show that eukaryotic communities in the gut are less diverse than in aquatic and terrestrial habitats, and few taxa are shared across habitat types, and diversity patterns of eukaryotes are correlated with those observed for bacteria. These results outline the distribution and diversity of microbial eukaryotic communities in the mammalian gut and across environments.

Keywords: protist, microbial ecology, microbial diversity, salinity, host-associated eukaryotes, parasites, intestinal protozoa, human microbiome

INTRODUCTION

A rich understanding of the distribution of microbial diversity across environments has emerged from high-throughput sequencing studies in the past decade. These studies have described many spatial and temporal patterns of variability within environments and have defined the major divisions in microbial community composition (Nemergut et al., 2013). Salinity represents the primary division among environmental samples for bacterial and archaeal communities (Lozupone and Knight, 2007; Auguet et al., 2010; Wang et al., 2011), while the vertebrate gut has the most distinct bacterial communities (Ley et al., 2008b). Studies characterizing microbial diversity deeply across hundreds

to thousands of samples are now common for bacteria (e.g., the Human Microbiome Project, the Earth Microbiome Project, MetaHIT), but are just beginning for microbial eukaryotes (Tara Oceans, ICOMM, BioMarks). As a result, progress characterizing the distribution of protist diversity lags behind our knowledge of bacteria, but morphological surveys (Larsen and Patterson, 1990; Patterson, 1996; Foissner, 2006; Weisse, 2008) combined with recent molecular data (Amaral-Zettler et al., 2009; Caron, 2009; Baldwin et al., 2013; Bates et al., 2013) provide a foundation of knowledge on the biogeography of protists across environments.

Our understanding of the diversity and function of host-associated microbial communities has grown exponentially in recent years, fueled by high-throughput sequencing and motivated by the realization that microbes have a profound influence on their host (McFall-Ngai et al., 2013; Sommer and Backhed, 2013). There are many commonalities in the bacterial taxa that comprise the microbiota across mammals, with the phyla Bacteroidetes and Firmicutes being predominant components (Ley et al., 2008b; Muegge et al., 2011). Overall, the mammalian gut harbors lower bacterial diversity and fewer phyla-level taxa than other environments (Ley et al., 2006). Across mammals, microbiota composition varies according to host phylogeny and diet (Ley et al., 2008b; Russell et al., 2014), and the composition of the human microbiota resembles that of our primate relatives (Ley et al., 2008b). Within humans gut microbiota is influenced by diet, health status, and age (Fierer et al., 2012; Lozupone et al., 2012). In addition, adoption of a western lifestyle, characterized by diets rich in processed food, antibiotic usage, and hygienic habits, has a particularly strong influence on the microbiota (De Filippo et al., 2010; Yatsunenko et al., 2012; Ursell et al., 2013). Diversity of the human bacterial microbiota has clearly declined in Western populations compared to populations with traditional agrarian lifestyles (De Filippo et al., 2010; Cho and Blaser, 2012; Lozupone et al., 2012; Yatsunenko et al., 2012).

Progress characterizing the eukaryotic component of the mammalian microbiome lags behind bacteria because high-throughput sequencing based investigations into the diversity of the mammalian microbiota have focused almost exclusively on bacteria (Parfrey et al., 2011; Andersen et al., 2013). The mammalian intestinal tract is home to many eukaryotes, including animals (e.g., helminths) and protists (e.g., amoebae and flagellates), and these taxa have been investigated for decades from a parasitological point of view with microscopy and targeted molecular approaches (Bogitsh et al., 2005). Studies of the eukaryotic component of the mammalian microbiota from a community perspective are beginning to come online, though many questions remain to be investigated (Andersen et al., 2013). Although sample sizes are generally small to date, these studies have shown that anaerobic fungi are dominant in mice (Scupham et al., 2006). Western human fecal communities include *Blastocystis* (Scanlan and Marchesi, 2008) and fungi (Dollive et al., 2012), while a survey of a single African individual revealed higher microbial eukaryote diversity (Hamad et al., 2012). The diversity of the eukaryotic microbiota in the human gut has not yet been systematically investigated from a community perspective in non-western populations. These populations provide an important perspective for understanding the eukaryotic microbiota that humans have co-evolved with over millions of years.

Eukaryotic microbes in the gut are generally considered parasites, and have long been recognized to contribute to host morbidity and mortality (Bogitsh et al., 2005). However, many are commensal (Bogitsh et al., 2005), or play beneficial roles as probiotics (McFarland and Bernasconi, 1993) or cellulose degraders (Kittelmann and Janssen, 2011). Further, increasing evidence suggests that eliminating the diverse microbial community that co-evolved with mammals over millions of years is detrimental to host health (Cho and Blaser, 2012; Lozupone et al., 2012), in support of the Old Friends Hypothesis (or hygiene hypothesis) (Rook, 2012). Eukaryotic microbes were part of our ancestral gut community and intestinal helminths were nearly universal (Goncalves et al., 2003). In humans, the transition to modern lifestyles is associated with dramatically lower diversity and prevalence of intestinal helminths, and with a rise in the prevalence of autoimmune disease (Rook, 2012). Yet, we know little about their role in healthy people. Recent analyses of common protists in the gut suggests that they may be part of the healthy microbiota in humans (Petersen et al., 2013).

Here, we use high-throughput sequencing to characterize eukaryotic communities found in the vertebrate gut from a diverse collection of mammalian fecal samples, including humans from the US and from remote communities in Malawi. To provide a broader context for understanding of the diversity of microbial eukaryotes in the gut, we also characterized a collection of samples from a wide range of other environments, including human skin, marine water, freshwater, soil, and air. The bacterial communities in these samples were also characterized to enable comparison of eukaryotic and bacterial biodiversity. In order to gain deeper insight into the distribution of eukaryotic diversity, we curated the SILVA reference database (Pruesse et al., 2007) so that both the taxonomy assigned to reference sequences and the phylogenetic tree constructed from these reference sequences reflects current knowledge. Eukaryotic environmental sequences are placed within this explicit phylogenetic context and assess the distribution of eukaryotic clades across environments.

METHODS

SAMPLE SET

We selected 185 samples that span a wide range of environments in order to assess broad patterns in eukaryotic communities (Table S1). The dataset analyzed here was chosen to include individuals from geographically diverse populations with contrasting lifestyles to enable testing the hypothesis that the transition to modern, highly hygienic lifestyles are correlated with low levels of diversity of eukaryotic microbes. We included samples from 23 individuals that reside in agrarian communities in Malawi that follow traditional lifestyles and 16 samples from 13 individuals residing in the US (Boulder, CO and Philadelphia, PA) and follow modern lifestyles (**Table 1**). Three individuals from Boulder were sampled at two time points 2 months apart (Costello et al., 2009). The US populations live in urban or suburban areas, consumed typical western diets, and did not report any health problems at the time of sampling (Costello et al., 2009; Yatsunenko et al., 2012). Individuals from populations in Malawi ate diets rich in

Table 1 | Human fecal samples.

Sample name	Village[a]	Age (years)	Original study[b]	Total seqs	Filtered seqs[c]	*Blastocystis*	*Entamoeba*
h101M	M: Mbiza	24.4	2	1041	982	ST3	*coli, hartmanii*
k57B.6Post	M: M: Mbiza	2.0	1	649	638	ST3	*coli*
h101A.4	M: Mbiza	2.3	2	719	546	ST3	
h101B.4	M: Mbiza	2.3	2	901	521	ST3	
k84M	M: Mayaka	30.6	1	821	493	ST1, ST3	*coli, dispar, hartmanii*
h186M	M: Mayaka	31.6	2	400	367	ST1, ST3	*coli, hartmanii*
k26M.1	M: Mitondo	29.5	1	929	361	ST3	*coli, dispar, histolytica, hartmanii*
h186A.1	M: Mayaka	2.0	2	1024	305		*coli*
h146B.2	M: Mayaka	1.7	2	319	261	ST2, ST3	*hartmanii*
h146M	M: Mayaka	33.5	2	246	233	ST1, ST3	*hartmanii*
m55M	M: Mbiza	adult	1	277	229	ST1, ST2	*coli, hartmanii*
k57M	M: Mbiza	30.8	1	233	212	ST1, ST2, ST3	*coli*
k80M	M: Mayaka	27.2	1	256	168	ST1	*coli*
m55S	M: Mbiza	child	1	263	165	ST2	*coli, hartmanii*
h47M	M: Chamba	adult	2	526	144	ST2	*coli*
k80A.7	M: Mayaka	1.9	1	285	118		*dispar, histolytica*
k84A.1	M: Mayaka	0.9	1	774	45	ST1, ST2, ST3	
h186B.1	M: Mayaka	2.0	2	900	44		*hartmanii*
h146A.2	M: Mayaka	1.7	2	935	10	ST3	*hartmanii*
h18A.3	M: Chamba	1.1	2	1028	8		*coli*
h47A.1	M: Chamba	0.6	2	1032	6		
h47B.1	M: Chamba	0.6	2	400	3		
h18B.5	M: Chamba	1.6	2	193	1		
USBldChld5	U: Boulder	3	2	485	123		*coli*
USchp60Mom	U: Philadelphia	33	2	1006	47		
USchp18Child	U: Philadelphia	3	2	977	35		
USBldChld8	U: Boulder	1.6	2	671	29		*coli*
M22Fcsw	U: Boulder	adult	3	935	14		
USBldChld4	U: Boulder	6	2	1159	7		
M21Fcsw	U: Boulder	adult	3	825	5		
USBldChld10	U: Boulder	1.3	2	913	1		
USBldChld2	U: Boulder	4.5	2	492	0		
USchp33ChildA	U: Philadelphia	5	2	378	0		
USchp33Mom	U: Philadelphia	45	2	781	0		
F11Fcsw	U: Boulder	adult	3	139	0		
M11Fcsw	U: Boulder	adult	3	502	0		
M23Fcsw	U: Boulder	adult	3	156	0		
M24Fcsw	U: Boulder	adult	3	221	0		
M31Fcsw	U: Boulder	adult	3	269	0		

[a] *Country where village is located: M, Malawi and U, USA.*

[b] *Original study: 1 = Smith et al., 2013; 2 = Yatsunenko et al., 2012; 3 = Costello et al., 2009.*

[c] *Filtered sequences have the following removed: Bacteria, Archaea, non-18S rDNA, mammalian DNA, plants.*

maize, legumes, and other plants (Table S1 from Yatsunenko et al., 2012) and were healthy and well-nourished at the time of sampling (Yatsunenko et al., 2012; Smith et al., 2013). These samples have been described in detail previously and bacterial diversity was previously reported (Costello et al., 2009; Yatsunenko et al., 2012; Smith et al., 2013). In addition, we included 22 samples from other mammals, also previously described and characterized for bacteria (Ley et al., 2008a; Muegge et al., 2011), to gain insight into the diversity of eukaryotic human microbiota relative to other mammals. Collection of the human fecal samples for these previously published studies was done according to protocols approved by Human Research Committees at the institutions involved which allow samples to be used for further research. De-identified DNA was sent to the University of Colorado for amplification. Collection of skin and oral samples was approved by the University of Colorado Human Research Committee (protocol 0109.23), which allows the samples to be used for further research. Finally, we included samples from wide variety of environments, many of which have been previously characterized for bacterial or fungal communities (Table S1). These include

air sampled over terrestrial environments (Bowers et al., 2011a, 2012), soil (Lauber et al., 2009; Ramirez et al., 2010; Eilers et al., 2012), freshwater (Shade et al., 2012), marine water, lichens (Bates et al., 2011), leaf litter (McGuire et al., 2012), and human oral and skin samples (Costello et al., 2009; Verhulst et al., 2011). The sequence data and MiMARKs (Yilmaz et al., 2011) compliant metadata is available for this study at the QIIME database (http://www.microbio.me/qiime/: study #1519 for eukaryotes and #1517 for bacteria) and at EBI (accession numbers ERP006039 and ERP005135).

MICROBIAL COMMUNITY CHARACTERIZATION

Sequences were PCR amplified with primers 515f and 1119r (Bates et al., 2012). The forward primer 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) is 3-domain universal and 1119r (5′ GGTGCCCTTCCGTCA 3′) is targeted toward eukaryotes. Primer specificity to eukaryotes and predicted amplification efficiency of eukaryotic lineages was assessed with the taxa coverage module in PrimerProspector (Walters et al., 2011). This program assesses the complementarity between the primer sequence and a reference database, in this case SILVA 111, and assigns a score based on the number of mismatches or gaps between the primer sequence and the reference, and mismatches as the 3′ end of the primer are more heavily penalized (http://pprospector.sourceforge.net/tutorial.html). Taxa coverage was assessed at three thresholds corresponding to three levels of specificity (Table S2). A threshold of 0.5 is predicted to generate efficient amplification and allows up to one mismatch at the 5′ end of the primer. The threshold of 1 allows one mismatch at the 3′ end of the primer or two mismatches in other primer regions, and threshold 2 allows 2–5 mismatches at the 3′ or 5′ ends of the primer respectively and amplification is expected to be poor or non-existent. This primer pair has high predicted specificity to eukaryotes, matching 86–90% of eukaryotic sequences but less than 0.5% of bacterial and archaeal sequences at a threshold of 0.5 and 1, respectively (Table S2). Many of the taxa expected to be in the mammalian gut based on parasitological studies are predicted to amplify well, including *Dientamoeba*, *Entamoeba*, *Blastocystis*, *Balantidium*, parabasalids, and nematodes (Table S2). However, there are two mismatches between the *Giardia* 18S sequence and the reverse primer suggesting a low efficiency (Table S2).

DNA was extracted with the MoBio PowerSoil kit following EMP standard protocols. PCR amplification was done in triplicate with an annealing temperature of 50C for 40 cycles. These permissive conditions were used to amplify the broadest range of eukaryotic taxa. Quantitation and pooling were done according to EMP standard protocols. The final pool was sent to Roche Core Facility. The libraries were amplified, sequenced and processed at the Roche Core Facility. Amplification was done according to the emPCR Amplification Method Manual—Lib-A LV GS FLX Titanium Series with the following edits for long amplicons. Using the Titanium Lib-A emPCR kit, the emulsions were made with A beads and A amp primers only and the following reagents: 1050 μL MBGW, 1500 μL emPCR additive, 860 μL 5× amplification mix, 300 μL Primer (A), 200 μL Enzyme mix, and 5 μL PPiase. The cycling conditions were 4 min at 94C followed by 50 cycles of 30 s at 94C and 10 min at 60C, ending with a hold at 10C. The library was then run as a standard XL+ run. This FLX+ run was sequenced with the standard flow order (400 cycles of TACG nucleotide flows), following the instructions in the Sequencing Method Manual—GS FLX+ Series—XL+ kit, as can be found on the www.my454.com website.

DATA PROCESSING AND QUALITY FILTERING

Data processing was done at the Roche Core Facility according to the GS FLX System Software Manual modified to optimize performance for metagenomic amplicon sequences. In order to generate high quality data for amplicons metagenomic applications, the default pipeline was tuned to meet the data quality requirements of the QIIME pipeline. The data was processed using 26amp_sl1000 pipeline which has the following tuning steps modified: (1) vfScanLimit was increased from the default of 700 to 1000, (2) the valley filter setting vfTrimBackScaleFactor was increased from the default value by a factor of 0.5, and (3) the quality filter setting QscoreTrimFactor was modified from the default value to a more stringent value. The Amplicon pipeline template was used to generate the modified pipeline XML file with the rCAFIE algorithm turned on.

Usearch version 6.1 was used to screen sequence for chimeras (Edgar, 2010). Sequences were additionally filtered for quality using split_libraries within QIIME version 1.5.0 (Caporaso et al., 2010b). Quality filtering excluded sequences with an average quality score of 25 or lower, reads longer than 1200 bp or shorter than 200 bp and reads with more than 5 ambiguous bases. We found that sequence quality dropped off significantly toward the end of the read, so we employed a strategy truncating sequences when quality scores that fell below 25 in a sliding window of 50 bp. These truncated reads were retained as long as they passed other quality filters and these averaged 444 bp in length.

In order to quantify concordance in the diversity patterns of bacterial and eukaryotic communities we sequenced the bacterial communities as well as the eukaryotic communities. Bacteria were sequenced with the 515f/806r primers (Walters et al., 2011) on the Illumina GAIIx platform at Washington University. Bacterial data was processed using standard protocols within the QIIME database (www.microbio.me/qiime). Archaea are also amplified with this primer set, but were excluded from the analysis in order to focus on the eukaryote to bacteria comparison and because there were too few Archaea OTUs for meaningful comparison. Low abundance OTUs, those containing less than 0.05% of the total reads in the dataset, were filtered out as recommended for Illumina sequence data (Bokulich et al., 2013). The samples were filtered to only include those 113 samples that had at least 150 sequences per samples in the eukaryotic data, and of these, samples with fewer than 3000 sequences were excluded from the analysis. The full dataset was used for taxon-based analyses and all samples were rarefied to 3000 sequences per sample for diversity analyses.

OTU PICKING AND TAXONOMY ASSIGNMENT

Eukaryotic sequence reads from the 454 FLX+ run were clustered into OTUs with a 97% similarity threshold, which was chosen to minimize the impact of sequencing error in inflating OTU

numbers (Stoeck et al., 2010; Bates et al., 2013). Reads were clustered into OTUs according to the open reference protocol (http://qiime.org/tutorials/open_reference_illumina_processing.html) using UCLUST (Edgar, 2010) within QIIME. This involves first clustering reads against the curated SILVA 108 eukaryotic database clustered at 97%, and these OTUs inherited the reference taxonomy. Sequences that failed to assign to the reference dataset were then clustered at 97% *de novo* with UCLUST. Taxonomy was assigned to these *de novo* sequences in one of two ways in order to maximize the taxonomic information and reliability. First, taxonomy was assigned using BLAST against the SILVA 108 97% reference database with an *e*-value cutoff of e-100. In cases where the *e*-value was less than e-100 taxonomy was assigned using the RDP classifier trained with the SILVA 108 97% reference set at genus level. Taxonomy assignments were also confirmed in using the PR2 reference database (Guillou et al., 2013). The resulting OTUs were filtered to exclude bacteria, archaea, vertebrates (thus removing host DNA), and plants (to exclude dietary sources) as well as non-SSU rDNA sequences. Finally, singleton sequences were excluded from the analysis to reduce the likelihood of including PCR and sequencing artifacts. After filtering, we excluded samples from further analysis that had fewer than 150 eukaryotic sequences/sample. This left 3883 OTUs from 113 samples (out of 185 total samples), corresponding to 84,576 sequences. Downstream diversity analyses used data rarefied to 150 sequences per sample, and taxonomy plots used the full dataset. In order to take full advantage of this dataset we assessed the taxonomic composition of human gut samples falling below the 150 sequences per sample threshold. In this case, a taxon (OTU) was considered present if the OTU was represented by least 5 sequences in the sample in question.

Although 150 sequences per sample is a low number by high-throughput sequencing standards, this sequencing depth adequately captures the diversity present (**Figure S1**). Direct comparison of numbers of bacterial and eukaryotic taxa is not possible because two different sequencing platforms were used here and the number of sequences per sample is much lower for eukaryotes. However, we can compare the relative differences in alpha diversity between sample types for eukaryotes and bacteria respectively, and sequencing depth for both domains adequately sample diversity. Rarefaction curves of Faith's Phylogenetic Diversity metric level off by 150 sequences per sample, particularly for host-associated samples (**Figure S1**). Similarly, we have adequate sampling of bacterial diversity and rarefaction curves are leveling off by 3000 sequences per sample for host-associated samples (**Figure S1**).

A phylogenetic tree reflecting the current understanding of eukaryotic relationships was constructed using the curated SILVA alignment as a template and the SILVA 108 tree as a constraint on the backbone relationships (see SILVA curation below). The representative set of sequences from this study was first aligned to the SILVA 108 97% representative set with PyNAST (Caporaso et al., 2010a). Representative sequences for each of the 3883 OTUs that aligned to the SILVA reference alignment were used to build a phylogenetic tree for diversity analysis and to assess patterns of phylogenetic groups by environment. The resulting alignment was dynamically filtered to remove the 10% most entropic positions and positions with greater than 95% gaps. This alignment was then used to build a phylogenetic tree with the topology constrained to the SILVA 108 97% tree (see below) in RAxML (Stamatakis, 2006). This tree was used for visualization in TopiaryExplorer (Pirrung et al., 2011), which allows branches to be colored according to sample metadata or taxonomy. The *p*-test from Martin (2002) and UniFrac test (Lozupone and Knight, 2005) were performed on the tree to assess whether the distribution of sequences from particular environments across the tree were significantly different than random, implemented in the beta_significance script within QIIME. In order to visually compare the diversity in the vertebrate gut to other environments, we filtered the tree to include equal sample numbers and equal (rarefied) sequences per sample. This was done by first filtering the OTU table to include the 32 fecal samples with more than 150 sequences per sample and a subsampled set of 32 environmental samples spanning the range of environments, and then rarefied to 150 sequences per sample for both eukaryotic 18S and bacterial 16S. This normalized OTU table was used to filter tips from the 16S and 18S trees.

Diversity analyses were carried out in QIIME using data rarefied to 150 sequences per sample for eukaryotes and 3000 sequences per sample for bacteria. The differences in rarefaction level are a result of the different sequencing platforms used for these datasets. Phylogenetically informed analyses of alpha and beta diversity [phylogenetic distance and unweighted UniFrac (Lozupone and Knight, 2005), respectively] utilized the tree described above. Non-phylogenetic beta diversity metrics performed poorly because very few OTUs were found across multiple sample types (**Table 2**). Unweighted UniFrac distance matrices were used in Analysis of variance tests (ANOSIM) to assess statistical differences across environments within QIIME. To assess the impact of unbalanced numbers of samples across habitat types, we randomly subsampled the dataset to include equal numbers of samples from each environment and then recalculated diversity metrics and performed ANOSIM tests. This procedure was repeated 1000 times. We visualized the differences in beta-diversity across sample types with non-metric multidimensional scaling (NMDS) plots, which were constructed in the software Primer E (Clarke and Gorley, 2006).

Table 2 | Proportion of shared eukaryotic OTUs.*

Environment	Fecal	Skin	Terrestrial	Freshwater	Marine
Fecal	190	1	3	1	0
Skin	1	68	34	6	1
Terrestrial	3	34	1796	80	2
Freshwater	1	6	80	354	4
Marine	0	1	2	4	482
Total OTUs	190	68	1796	354	482
% Unique	97%	38%	93%	74%	99%

**Calculations were done based on the full dataset, and exclude fungi. Fungi have low taxonomic resolution for 18S rRNA (Schoch et al., 2012), thus shared fungal 97% OTUs may be quite divergent.*

We took advantage of the long sequence reads from the 454 FLX+ to further investigate the phylogenetic position of *Entamoeba* and *Blastocystis*, the two most common taxa detected in the gut. We aligned *Entamoeba* and *Blastocystis* representative sequences to the reference taxa from the PR2 database, and then constructed maximum likelihood phylogenies with RAxML. These trees were constrained to the reference phylogeny for these clades, which was derived from the literature (Stensvold et al., 2011; Alfellani et al., 2013). The placement of *Entamoeba* and *Blastocystis* sequences was used to confirm the taxonomic identities of these OTUs (**Table 1**).

CURATION OF THE SILVA EUKARYOTIC DATABASE

The SILVA 108 ribosomal database (Pruesse et al., 2007) was downloaded from SILVA (http://www.arb-SILVA.de/). Sequences were initially filtered to remove unclassified environmental sequences. The remaining ∼55,000 sequences were dereplicated by clustering at 97% with UCLUST, resulting in ∼11,000 sequences. A representative set was then chosen for these OTUs based on the longest sequence. The filtered out environmental sequences were then clustered against the representative set of 97% OTUs using UCLUST ref within QIIME. Those sequences that did not match the reference dataset were then clustered at 97% *de novo* and the longest representative sequence chosen for each cluster. This resulted in a final SILVA eukaryotic 97% representative set with 14,236 sequences.

The 97% reference dataset was aligned with PyNAST (Caporaso et al., 2010a) in QIIME with a threshold of 70% similarity and a template alignment from Katz et al. (2011) [TreeBase study 11336, matrix M8584; (Katz et al., 2011)]. The resulting alignment was dynamically filtered to remove the 20% most entropic positions and positions with more than 90% gaps. A phylogenetic tree was constructed with RAxML version 7.3.0 (Stamatakis et al., 2008), using the tree topology from the multigene study of Parfrey et al. (2010) with updates based on subsequent papers (e.g., Adl et al., 2012) as a constraint.

The database taxonomy was curated to reflect current views of eukaryotic taxonomy and maximize the taxonomic information available for environmental sequences. Major clade information was added based on Parfrey et al. (2010) and Adl et al. (2012). To maximize the informativeness of the SILVA data set, high-level taxonomy was assigned to uncultured environmental sequences by placing these uncultured reads into the tree of SILVA representative sequences with the RAxML EPA algorithm (Berger et al., 2011) and assessing their position in a phylogenetic tree. Sequences that were nested within clades were assigned taxonomy based on that clade at a high level (e.g., Ciliate or Fungi). Sequences that were mislabeled (i.e., sequence labeled as fungi that fell within the plants) were identified in the tree, confirmed by BLAST and then removed from the representative set. The curated SILVA 108 database is available at http://qiime.org/home_static/dataFiles.html.

RESULTS AND DISCUSSION

EUKARYOTIC DIVERSITY IN THE HUMAN GUT

Eukaryotic microbes are common components of the human gut microbiota in healthy individuals. *Blastocystis*, *Entamoeba*, trichomonads, and yeast were frequently detected in human gut samples (**Figure 1**). Closer inspection of the taxa reveals that most are likely commensal rather than pathogens. For example, *Entamoeba* was detected in both populations. While the genus *Entamoeba* includes *E. histolytica*, the causative agent of the deadly amoebic dysentery (Bogitsh et al., 2005), the vast majority of *Entamoeba* sequences detected here fall within the commensal species *Entamoeba coli, E. dispar,* and *E. hartmanni* (**Table 1**). *Entamoeba histolytica* was detected in low abundance in two individuals that also harbored *E. dispar*.

Blastocystis was abundant in many samples (**Figure 1**), and represented by subtypes ST1, ST2, and ST3 (**Table 1**). Historically, *Blastocystis* has been considered a pathogen and it is associated with Irritable Bowel Syndrome (Yakoob et al., 2010; Poirier et al., 2012). However, the clinical importance of *Blastocystis*, its pathogenicity, and variation in pathogenicity among subtypes, is widely debated (Tan et al., 2010; Coyle et al., 2012; Scanlan and Stensvold, 2013). Some evidence suggests that *Blastocystis* is a normal component of the microbiota in many individuals—perhaps even a beneficial component—as it has been detected at high prevalence in healthy people (Scanlan and Marchesi, 2008; Petersen et al., 2013; Andersen et al., submitted), its presence is negatively correlated with intestinal disease (Petersen et al., 2013), but see Cekin et al. (2012). High prevalence of *Blastocystis* has been reported in other epidemiological studies of African countries, up to 100% reported in a Senegalese cohort, half of which had no gastrointestinal symptoms (El Safadi et al., 2014). Many other taxa that populate parasitology textbooks were also detected at lower levels, including *Chilomastix*, nematodes, and other parabasalids. We do not detect common gut symbionts such as *Dientamoeba* (Parabasalia), *Cryptosporidium* (Apicomplexa), or *Giardia* (Diplomonadida). The primers used here are a poor match for *Giardia* (Table S2) and may have failed to amplify *Giardia* DNA. The primers are predicted to work well with *Cryptosporidium*, but our DNA extraction method (bead beating rather than freeze thaw cycles) may have been insufficient to break open the robust spores of *Cryptosporidium* (and similar problems may further hinder our ability to detect *Giardia*). *Dientamoeba* is also predicted to amplify with our primers (Table S2). While prevalence is generally quite high in Europe, *Dientamoeba* prevalence is variable worldwide and generally low (less than 5%) in Africa (Barratt et al., 2011). However, specific diagnostic assays would be necessary to rule out presence of these taxa with any confidence.

We assessed eukaryotic diversity across two geographically distant populations whose inhabitants follow either traditional, agrarian lifestyles (Malawi) or modern, urban lifestyles (US). However, our ability to compare eukaryotic diversity across populations is hampered by low counts of eukaryotic sequences in US individuals and young children. Taxa presence above was calculated based on OTUs represented by at least five sequences in a given sample. In order to compare diversity across populations and across sample types more broadly, we filtered out samples with fewer than 150 eukaryotic sequences. While all but three human fecal samples had greater than 150 sequences per sample in total, 27 samples fell below this threshold after removing sequences from bacteria, host, and dietary plants. These

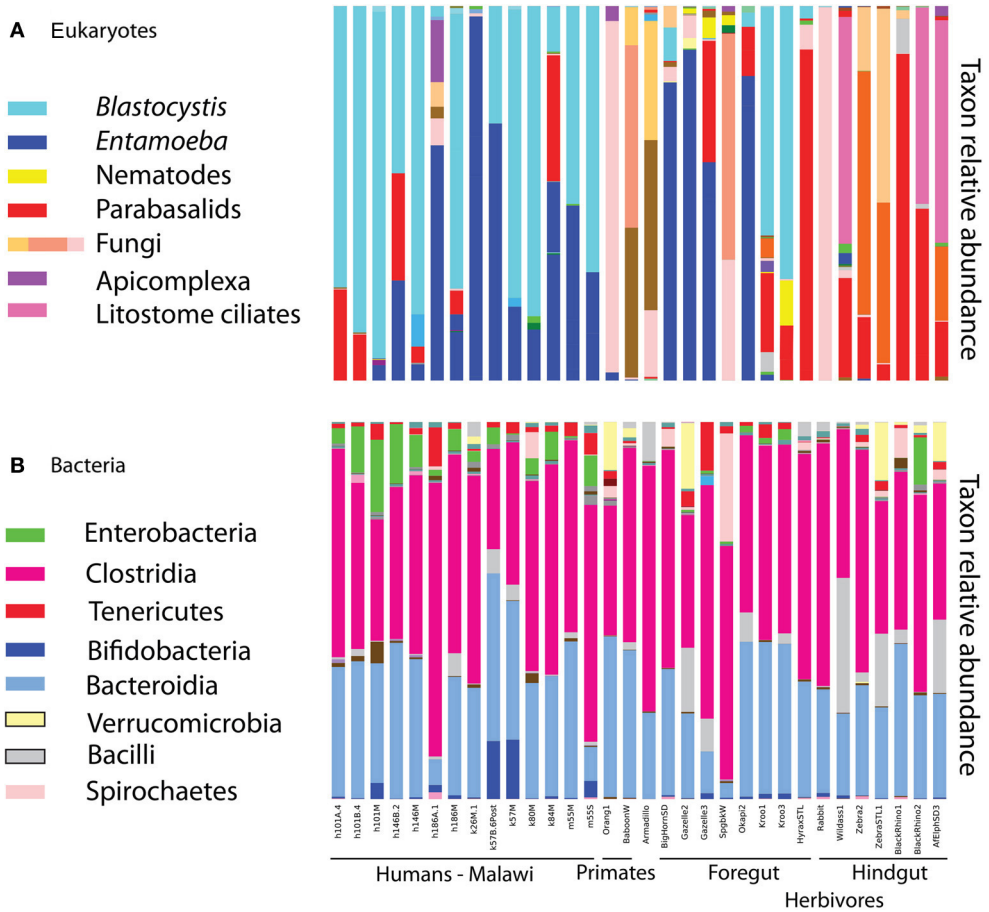

FIGURE 1 | Relative taxon abundance of mammalian (including human) fecal samples demonstrates heterogeneity in the presence of eukaryotic lineages across mammals, while the same bacterial lineages are consistently dominant. (A) Eukaryotes, **(B)** bacteria. Each bar represents an individual fecal sample from humans and other mammals, and only samples with at least 150 sequences in the 18S are represented.

non-target taxa account for 94–100% of the sequences from all but one US samples and most children age two and younger (**Table 1**). One samples from a three-year-old US child had a large portion of sequences derived from *Entamoeba coli*. The primer set used here targets eukaryotic 18S has a low affinity for vertebrate 18S sequences, and successfully amplified the eukaryotic community in most samples, including environmental samples and mammalian feces (Table S1). We suspect that the high proportion of non-target sequences amplified in samples from the US and from small children reflects a lower eukaryotic biomass and/or diversity in these samples. This hypothesis requires further investigation, but is inline with other results. Previous studies report lower bacterial diversity in western populations and in young children (reviewed in Lozupone et al., 2012). Further, lower prevalence of gut symbionts is associated with the adoption of western lifestyles (Rook, 2012), and prevalence and diversity are lower in temperate regions compared to the tropics (Bogitsh et al., 2005; Harhay et al., 2010).

EUKARYOTIC MICROBIOTA IN THE MAMMALIAN GUT

Mammals as a whole harbor a diverse community of eukaryotic microbes in their gut, and compositional differences follow host phylogeny and diet. The human gut microbioes is similar that of other mammals, particularly of primates.

Diet drives differences in bacterial community composition across mammalian species (Ley et al., 2008b; Muegge et al., 2011). We also see compositional differences according to diet in the eukaryotic communities. Herbivores make up most of our mammalian samples that successfully amplified, and are differentiated between hindgut and foregut fermenters. The presence and absence of entire lineages varies according to dietary group, for example only hindgut fermenting herbivores harbor litostome ciliates and anaerobic fungi (e.g., *Neocallimastix;* **Figure 1**). Lineages that are present in multiple host species such as *Blastocystis* and *Entamoeba* show species level divergence that tracks host phylogeny. Artiodactyls harbor *Entamoeba bovis,* while primates have *Entamoeba coli* and *E. hartmanii* (Table S1). Host-specificity is also observed in the distribution of *Blastocystis* subtypes (Table S1). We detected *Blastocystis* ST1, ST2, and ST3 in humans (**Table 1**) and also in the primates (baboon and orangutan) (Table S1). Kangaroos, foregut-fermenting herbivores, had large numbers of *Blastocystis* ST8 (**Figure 1**; Table S1).

Diversity patterns for eukaryotic microbes within the mammalian gut differ in two ways from those of bacteria. First,

eukaryotic microbes show a patchy distribution across samples, such that the most abundant lineages in some samples are completely absent from others (**Figure 1**). In contrast, bacterial community composition at comparably high taxonomic levels is broadly consistent across individuals and across populations; e.g., Bacteroidetes and Firmicutes are generally the dominant phyla (**Figure 1**; Ley et al., 2008b; Consortium, 2012; Yatsunenko et al., 2012). Second, within a phylum-level lineage there is less diversity at the strain and species level for eukaryotes, even after controlling for differences in sequencing depth (**Figure 2**). This suggests that presence or absence of deep lineages may be more informative than variation at lower taxonomic levels for eukaryotes.

DIVERSITY OF GUT MICROBIOTA COMPARED TO OTHER ENVIRONMENTS

The microbial eukaryotic communities detected in the mammalian gut are quite distinct from environmental communities both at the OTU level, as seen in the low numbers of shared OTUs (**Table 2**) and at higher taxonomic levels (**Figures 2**, **3**). Just 3% of non-fungal OTUs from the gut are shared with skin, terrestrial, and aquatic environments (**Table 2**). The composition eukaryotic communities in the mammalian gut is significantly different than the composition found in environmental samples (ANOSIM $p = 0.001$, $R = 0.76$), and this is true for bacteria as well (ANOSIM $p = 0.001$, $R = 0.94$). Overall, beta-diversity patterns observed for eukaryotes are significant similar to bacterial beta-diversity as assessed by Mantel tests comparing the unweighted UniFrac distance matrices ($p = 001$, $R = 0.658$; $N = 113$). The distinctiveness of gut communities can also be seen when the branches of the 18S and 16S trees are colored according to the environment where the sequences were detected (**Figure 2**). Sequences from the gut are significantly clustered in both 16S and 18S (**Figure 2**) as assessed by the phylogenetic test [p-test $p < 0.001$; (Martin, 2002)] and UniFrac significance test ($p < 0.001$).

In accordance with previous observations, fewer lineages of eukaryotes reside in the mammalian gut than in other habitats, and those lineages that have successfully colonized the vertebrate gut have diversified as they have co-evolved with their hosts over millions of years (Parfrey et al., 2011). Similar patterns have also been observed for bacteria (Ley et al., 2006). Here, we see significantly lower levels of alpha diversity in gut communities compared to other environments for eukaryotes (t-test comparing Faith's phylogenetic distance in the gut vs. environmental samples: $p < 0.001$), and bacteria ($p < 0.001$).

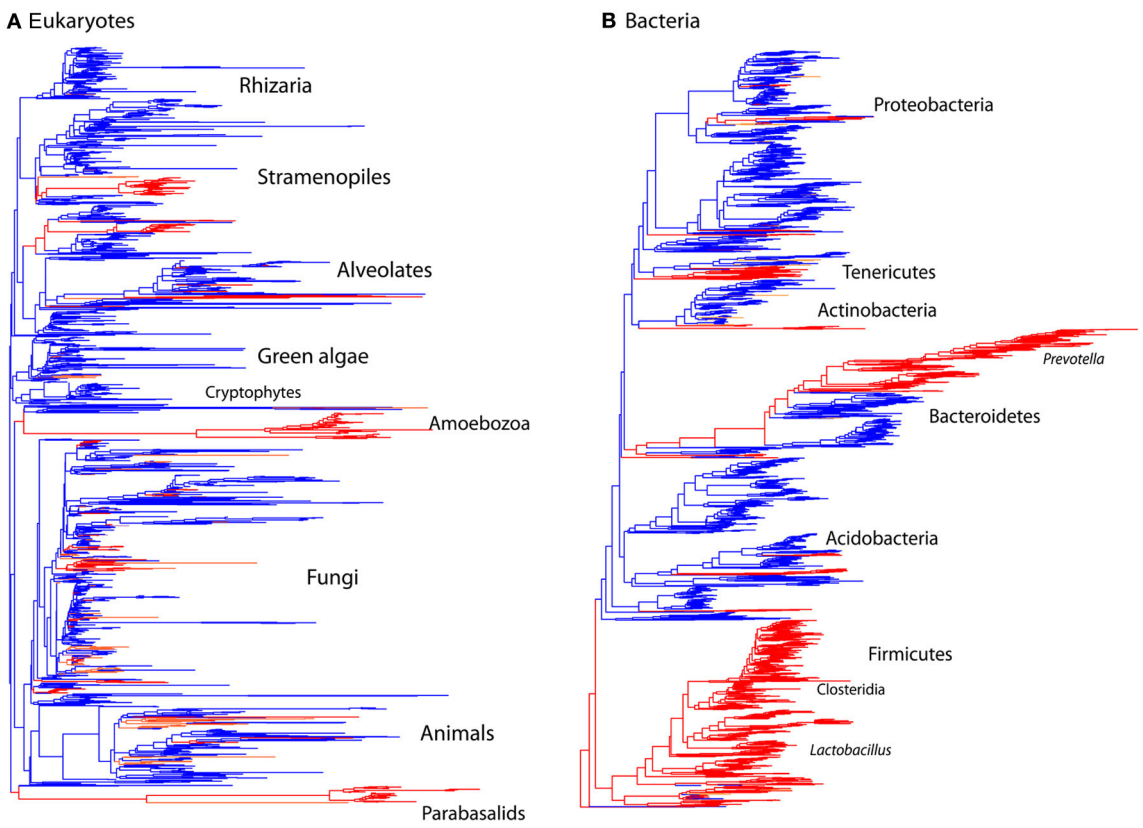

FIGURE 2 | Comparison of the phylogenetic distribution of taxa from mammalian gut to other environments. Sequences detected in the mammalian gut come from a smaller number of lineages and have lower overall diversity compared to other environments, reflecting the limited number of lineages that have successfully colonized animal hosts. Tree contains sequences from 32 mammalian gut samples (red) and 32 samples total from skin, terrestrial, and aquatic habitats (blue). Tips present correspond to the data rarefied to 150 sequences per sample for comparison. **(A)** Eukaryotic 18S rRNA tree constructed using RAxML with the topology constrained to the SILVA 108 reference tree. **(B)** Bacterial 16S rRNA tree from Greengenes 2011 release. Branches are colored according to the environment that contributed the majority of the sequences.

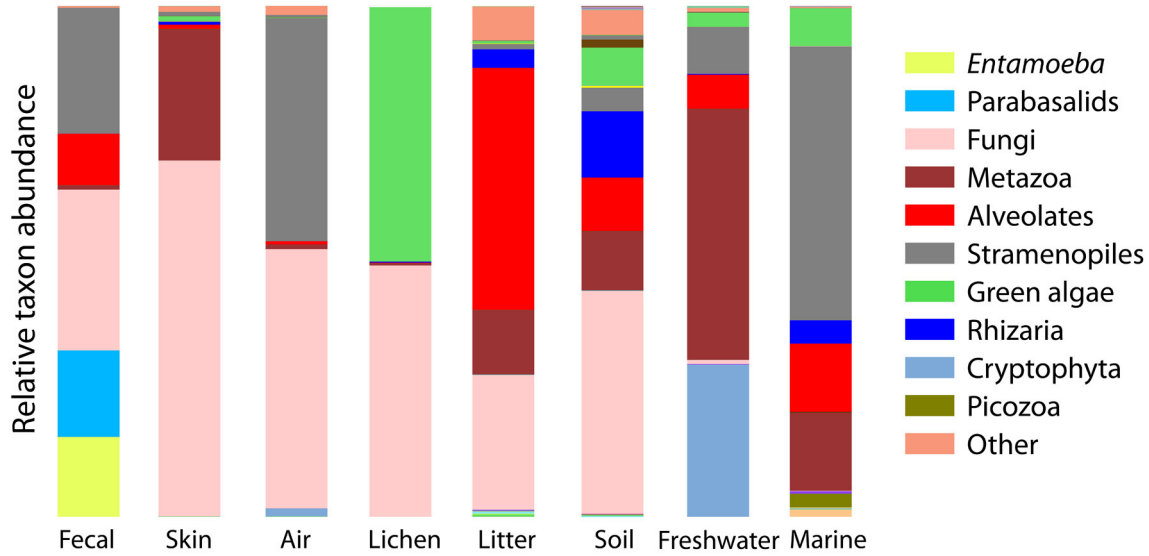

FIGURE 3 | Bar chart of the relative abundance of sequences falling into the major clades of eukaryotes depicts the overall divergence in community composition across sample types. Major clades are the deepest divisions within eukaryotes (Parfrey et al., 2010; Adl et al., 2012) and are roughly equal to the phyla or superphyla level of bacteria.

EUKARYOTIC COMMUNITIES ASSOCIATED WITH HUMAN SKIN RESEMBLE TERRESTRIAL SAMPLES

Eukaryotic communities associated with human skin are composed mostly of fungi and have low diversity overall, in line with expectations from other studies (Paulino et al., 2006; Findley et al., 2013). Skin samples group with terrestrial samples in NMDS plots of unweighted UniFrac (**Figure 4**). Similarity in the fungi detected in skin and terrestrial samples accounts for much of this similarity; 70% of the OTUs on skin are fungi, and of these more than 80% (113 OTUs) are shared with soil or other terrestrial samples. The low taxonomic resolution of fungi with the 18S marker may inflate the number shared OTUs to some extent (Schoch et al., 2012). Non-fungal OTUs detected on skin correspond to mites and a handful of low abundance OTUs that are commonly found in soil such as cercozoan flagellates. The overlap between skin and soil communities may reflect frequent contact between skin and soil, or with airborne microbes, which can have high abundances of soil-associated taxa (Bowers et al., 2011b). In support of this hypothesis, skin bacterial communities also frequently group with environmental samples (**Figure 4**). These results are suggestive, but are drawn from skin and soil samples taken in different locations within different studies (see Methods). Testing the hypothesis that skin communities resemble terrestrial environments because contact enables frequent dispersal requires samples from human skin and the surrounding environment, including dust and soil, collected at the same time.

COMPARISON OF EUKARYOTIC COMMUNITIES IN OTHER HABITATS

Our dataset includes samples from a range of environments and enables us to compare eukaryotic communities across environmental habitats. Microbial eukaryotic communities are highly differentiated across host marine, freshwater, and terrestrial habitats as assessed by ANOSIM (**Figure 4**; ANOSIM $R = 0.78$, $p = 0.001$). The sample set analyzed here includes more soil and other terrestrial samples, such as lichens and leaf litter than water samples (Table S1), but the differences across habitat types persist when the data is subsampled to equal sample numbers across habitat types (see Methods). For each of the 1000 sub-sampled trials, the divide between freshwater, marine, and terrestrial environments was highly significant and explains much of the variation (ANOSIM ranges: $p = 0.001$ to 0.005 and $R = 0.65$ to 0.60). These habitats were also significantly clustered in the 18S tree (p-test $p = 0.001$ for each pair of environments).

Beta-diversity differences across environments are underlain by a strong differentiation in the high-level clades present across environments (**Figure 3**). Some clades are restricted to one type of sample, for example, Amoebozoa (*Entamoeba*) and parabasalids are characteristic of fecal samples and cryptophytes comprise a large portion of the freshwater community, while the recently identified Picozoa clade (formerly "picobiliphytes"; Seenivasan et al., 2013) is restricted to marine environments. Yet, across all environments, diversity is dominated by just a few clades. Animals, fungi, alveolates, Cercozoa, and stramenopiles make up 79% of all sequences (**Figure 3**). At the OTU level very few taxa are shared across habitats (**Table 2**).

Communities from environmental samples show a distinct separation between terrestrial and water samples, and between marine and freshwater samples in beta-diversity plots (**Figure 4**). In accordance with previous studies that report salinity as the most important factor structuring bacterial and archaeal community composition (Lozupone and Knight, 2007; Auguet et al., 2010; Wang et al., 2011), and we also see a major divide in bacterial community composition between freshwater vs. marine habitats (**Figure 4**). Eukaryotic taxa also cross the saline/non-saline boundary infrequently (e.g., Shalchian-Tabrizi et al., 2008;

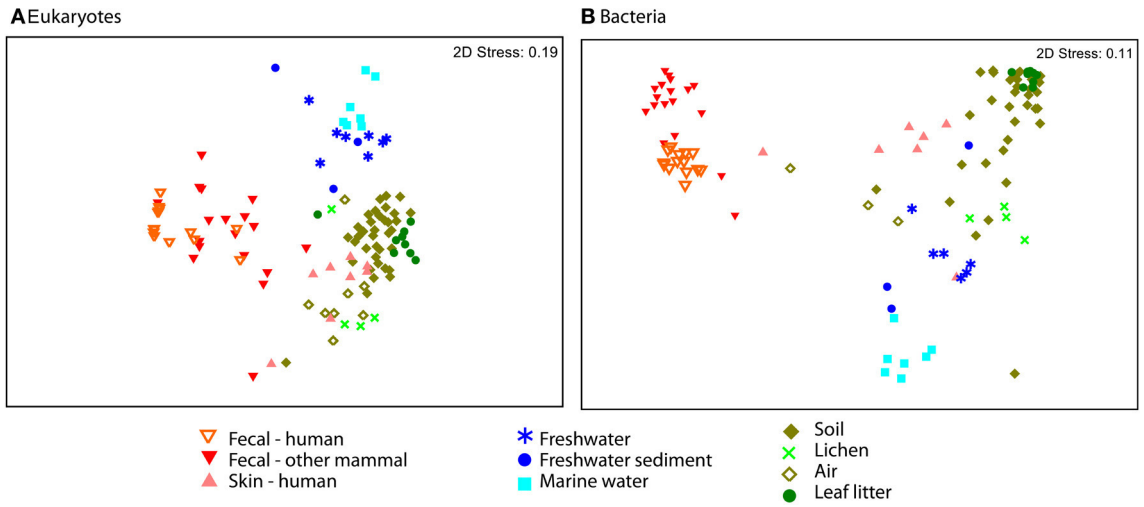

FIGURE 4 | NMDS plot of unweighted UniFrac reveal separation across major environmental categories. Plots **(A)** Eukaryotes and **(B)** Bacteria show the distinction between fecal samples (red and orange) and those from other environments, including skin (pink). Air samples were collected over terrestrial habitats.

Logares et al., 2009; Brate et al., 2010). In our data, compositional differences between freshwater and marine eukaryotic communities are highly significant (ANOSIM $p < 0.001$, $R = 0.58$), though our dataset includes a limited number of samples. Interestingly, the difference between aquatic and terrestrial environments are also significant and explain more variation in community structure (ANOSIM $R = 0.71$ for terrestrial vs. freshwater and $R = 0.85$ for marine vs. terrestrial comparisons). Further studies that include large numbers of samples from all three habitat types, preferably from consistent geographic locations, will be necessary to determine the deepest divisions in eukaryotic community composition across environments.

CONCLUSIONS

Our results demonstrate clearly that microbial eukaryotes are a normal component of the mammalian microbiota, and that the communities they form, although not as diverse as bacterial communities in the gut, are nonetheless diverse and correlate with key features of their hosts. Interestingly, humans with non-western diets and lifestyles are comparable to other mammals in the microbial eukaryote diversity they harbor. In contrast, humans living Western lifestyles instead have very low diversity of gut microbial eukaryotes. Whether these differences are due to diet, hygiene, level of contact with animals, host genetics, or other lifestyle factors that differ among the populations surveyed remains a topic for further work: of particular interest is whether the loss of the microbial eukaryote diversity with which we as mammals have co-evolved is a trigger for the autoimmune diseases that are far more prevalent in Western populations.

One intriguing difference between eukaryotic and bacterial communities is that eukaryotic communities in the vertebrate gut are heterogeneous across samples, whereas the dominant bacterial lineages are consistently recovered across individuals and across species. The patchy distribution of eukaryotes across individuals, combined with the host-species specificity of resident eukaryotic microbes, suggests that it will be difficult to clearly identify the healthy, or "normal," core eukaryotic microbiota of the human gut, just as is it is also difficult to identify a core gut bacterial community shared across humans (Li et al., 2013). Consequently, future studies of microbial eukaryote communities should focus more on identifying variation that is associated with different phenotypic states, including disease states.

Finally, comparison of the mammalian gut to other environments shows that fewer deep lineages are associated with the gut than in free-living communities, and alpha diversity is lower. This pattern resembles the pattern found in bacteria in the same environments. Eukaryotes have less diversification within lineages at shallow levels than observed for bacteria, however, suggesting that although the big picture of high-level diversification is the same across these taxa, the fine-grained patterns may differ. With the improved tools for eukaryotic surveys presented here, we are now poised to characterize microbial eukaryotes across environments on a large scale in projects such as the Earth Microbiome Project, providing a much richer understanding of the relationships between pathogens, commensals, and beneficial members of our microbial eukaryote community.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00298/abstract

Figure S1 | Rarefaction curves with alpha diversity metric PD Whole tree. Rarefaction curves are approaching an asymptote indicating diversity has been adequately captured, especially for fecal samples. Error bars are standard deviation. **(A)** Eukaryotes and **(B)** Bacteria.

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Microbiomes, plausible players or not in alteration of host behavior

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INTRODUCTION

Many parasites can affect the physiology and behavior of their hosts in ways that seem to improve the parasites' chances of completing their life cycle (Biron and Loxdale, 2013; Lafferty and Shaw, 2013; Webster et al., 2013). These parasite species are so-called "manipulative parasites." Common habitats of manipulative parasites are the host's body cavity, muscles and brain (Lafferty and Shaw, 2013). Typically the host's neural, endocrine, neuromodulatory, and immunomodulatory systems are targeted (Adamo, 2013; Lafferty and Shaw, 2013). In evolutionary biology, manipulation of host behavior by parasites is considered to be an example of the "extended phenotype" concept (Dawkins, 1982; Libersat et al., 2009). There are numerous fascinating cases of alteration of host behavior induced by a parasite; for instance, the suicidal behavior of crickets induced by hairworms (Thomas et al., 2002; Biron and Loxdale, 2013).

Many studies on strategies used by manipulative parasites assume that only two organisms are involved in crosstalk based on Dawkins' assumption: the host and a manipulative parasite. However, hosts are frequently invaded by more than one species of parasite (Ferrari and Vavre, 2011; Cézilly et al., 2014). The interests of different parasitic species may conflict; for example, two parasites may share an intermediate host but require a different definitive host. Parasite-parasite interactions in the intermediate host can result in perturbation of the parasite infection process for each parasite species (Lafferty and Shaw, 2013; Cézilly et al., 2014).

As far as we know, microbiomes are not considered to be taking part in crosstalk between an aquatic host and a manipulative parasite. Therefore, we first briefly present the background for microbiomes as plausible and underestimated players in the crosstalk in host-manipulative parasite associations in aquatic ecosystems, and secondly we discuss concepts and -omics methods to determine whether or not host microbiomes can influence host behavior in aquatic models. Finally, we discuss the importance of considering context-dependent changes in the analysis of -omics data to decode and understand the role of a host microbiome in the alteration of host behavior in aquatic ecosystems.

BACKGROUND: MICROBIOMES AND ANIMAL BEHAVIOR

Hosts contain distinct habitats where microorganisms and metazoan species like cestodes, nematodes, hairworms, trematodes, and acanthocephalan worms live and compete for resources. The ecological communities of commensal and symbiotic microorganisms (i.e., bacteria, yeasts, fungi, and viruses) living in the internal (example: gut, lachrymo-nasal, respiratory, and urogenital tracts) and epidermal (example: skin, fishes' gills) body surfaces of metazoans are typically considered "normal" or "healthy" microbiomes (Simpson et al., 2005; Mueller et al., 2012; Relman, 2012; Llewellyn et al., 2014). Pioneer studies on microbiomes were done on animal models (example: cow, honeybee, chicken, drosophila, mosquito, mouse, pig, teleost species, zebrafish) and humans (i) to identify microbiomes in healthy individuals; (ii) to decipher microbiome responses to host pathology, parasite invasion, host nutrition and host stress; and (iii) to determine plausible impacts of microbiomes on animal behavior (Smith et al., 2007; Ezenwa et al., 2012; Fagundes et al., 2012; Louis and Flint, 2013; De Palma et al., 2014; Llewellyn et al., 2014; Sison-Mangus et al., 2014; Stilling et al., 2014).

Most microbiome studies have focused on the gut microbiome, because this is a key host habitat for dynamic interactions between the animal host and components of its environment, including nutrients, liquids, and parasites. To date, these studies have revealed that the gut microbiome is involved in key host functions that assist the host in completing its life cycle: for example, (i) prevent parasite invasion of host tissues (example: helminthes, apicomplexa (malaria, sleeping sickness), Vibrio, *Pseudomonas, Streptotoccus*), (ii) nutrition (i.e., aid host digestion by producing molecules helping in food assimilation), and immunomodulation (i.e., stimulation of host immune system favoring an efficient immunity against invasive organisms) (Ringø et al., 1997; Gomez and Balcazar, 2008; Ley

et al., 2008; Louis and Flint, 2013; De Palma et al., 2014; Llewellyn et al., 2014; Stilling et al., 2014). Recent research suggests that the host gut microbiome is closely involved in the maturation and functioning of the central nervous system (CNS) of model species (i.e., human being and mouse) by producing and releasing neuroactive molecules (Cryan and Dinan, 2012). Moreover, ethologists observed that behaviors (example: mating, feeding, and anxiety) of many animal species including human beings could be altered by the host gut microbiome (Archie and Theis, 2011; Ezenwa et al., 2012; Lizé et al., 2013; Alcock et al., 2014).

MANIPULATIVE PARASITES AND MICROBIOMES

Microbiome research is new and mainly focuses on mice and human models (Fagundes et al., 2012; Alcock et al., 2014). However, recent work shows that microbiomes of teleost fish serve as defense against parasitic microorganisms, for example, by preventing the colonization of pathogenic bacteria (example: *Streptococcus* species) via competitive exclusion or via toxic secondary metabolites (Llewellyn et al., 2014). To date, no study suggests that microbiomes can alter the behavior of aquatic hosts but it was demonstrated that a part of the microbiome (i.e., bacterial biofilms) of many marine invertebrates, from corals to sea urchins, play a key role in the settlement behavior of larval stages (Ezenwa et al., 2012; Huang et al., 2012). The understanding of molecular tactics used by microorganisms to modulate the behavior of their hosts results mainly from studies of host-manipulative parasite associations. Many animals use behavioral strategies to avoid manipulative parasite species. Manipulative parasites manipulate host behavior by secreting molecules that act directly and/or indirectly on the maturation and functioning of host CNS (Biron and Loxdale, 2013; Hughes, 2013). However, given the diversity of non-pathogen and beneficial microorganisms in aquatic ecosystems, it is important to expand the view of host behavior/microorganism interactions to include at least the gut microbiome as a third plausible player when a manipulative parasite interacts with its host because the gut microbiome produces neuroactive molecules that can pass via the enteric nervous system (ENS) to interact with the host brain and host nervous system (Gershon, 2008; Fagundes et al., 2012; Schoofs et al., 2014). Thus, cross-talk could be engaged between at least three groups of organisms: the host and its gut microbiome, and an invading manipulative parasite species (**Figure 1**).

When manipulative parasites are not living in the host's CNS, the mechanisms mediating host behavioral changes are more difficult to determine. Secretion of effective amounts of neurotransmitters by parasites is not easy to confirm (Adamo, 2013). Gammarids may be one example (Maynard et al., 1996; Helluy, 2013). This crustacean family is an intermediate host of many manipulative parasite species (example: trematodes and acanthocephalan worms) (Ponton et al., 2006; Lefèvre et al., 2009; Biron and Loxdale, 2013). The molecular mechanisms used by these parasites (i.e., trematodes and cestodes) to manipulate the host biochemical pathways to alter the host's serotonergic system are still unknown.

Because gut microbiomes can produce neuroactive molecules, we assume that when a manipulative parasite, for instance *Polymorphus paradoxus*, (Acanthocephala: Polymorphidae) invades the gut of *Gammarus lacustris* (Amphipoda, Gammardiae), a part of the gut microbiome would respond to the invasion. Assuming that it is likely that microbiome responses to parasites are conserved between aquatic and terrestrial species, microbiome immune molecules and host immune strategies will target the parasite. During this host/microbiome/manipulative parasite crosstalk, neuroactive molecules produced by the gut microbiome should have additive and/or negative effects during the manipulative process by *P. paradoxus*. Such an effect could occur via a disturbance of

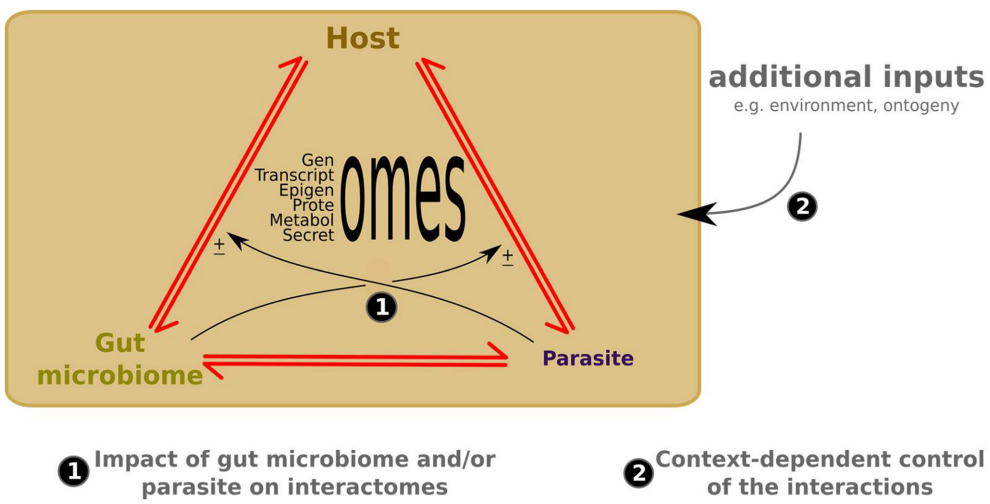

FIGURE 1 | New -omics and meta-omics technologies help to identify candidate molecular interactions in the three-partner crosstalk (1) and context-dependent control of the interactions (2). Red arrows indicate molecular crosstalk between interacting partners; ± refers to possible additive, antagonist or neutral effects of the gut microbiome or parasite on the host/parasite or host/gut microbiome crosstalk, respectively.

the host ENS and/or via the microbiome neuroactive molecules released into the host's hoemcoel. The possible additive effect of the gut microbiome could help answer the key questions regarding how parasites manipulate their hosts and how small metazoan parasites produce enough neuroactive molecules to alter directly or indirectly the host CNS functioning? Whether or not the gut microbiome is involved in these interactions is a fascinating question. This new and promising research avenue will contribute to our general knowledge of molecular crosstalk in host/gut microbiome/parasite relationships and may assist in the search for new methods to treat parasitic diseases.

In order to study host-manipulative parasite associations, there are key experimental steps needed in order to decipher the possible host/gut microbiome/manipulative parasite cross-talk (**Figure 1**): (i) sampling of host CNS, host ENS, host GM and of the manipulative parasite from laboratory strains and/or from field sampling collection for infected and uninfected hosts (i.e., control) before, during and after manipulation by the parasite; (ii) use of complementary -omics tools (example transcriptomics, proteomics and metabolomics) to reveal the host/gut microbiome/manipulative parasite cross-talk before, during and after the manipulation by the parasite; (iii) analysis of -omics results with specialized software including genome/environment statistical methods to find candidate molecules; (iv) functional analysis (microinjection, immunochemistry, RNAi) and interactome bioassays to confirm or determine the key roles (or not) of the candidate molecules from the three organisms in interactions, and to establish a kinetic map of the biochemical networks of molecules involved in the host/gut microbiome/manipulative parasite cross-talk by using software like cytoscape (http://www.cytoscape.org/). These "-omics" guidelines could help to suggest when the gut microbiome could have additive, antagonist or neutral effects during the manipulation process of a host by a manipulative parasite.

Biological entities named interactomes correspond to the complete set of protein–protein interactions existing between all of the proteins of an organism (Biron et al., 2006). The identification of protein interactions and protein complexes is being increasingly refined in many single and multicellular organisms (Bouveret and Brun, 2012; Braun and Gingras, 2012). However, little is known about large-scale protein interactions between hosts and parasites, and nothing is known about the possible host/gut microbiome/manipulative interactome, although the drawing up of such maps will provide an essential foundation to determine the success or not of molecular strategies used by manipulative parasites to take control of many host cellular functions, and to alter the behavior of their host, which should favor and ensure the continuation of their life cycle.

IMPACT OF CONTEXT-DEPENDENT CHANGES

Microbiome interactions may be context-dependent. For example, if hosts have resistant or susceptible genotypes and parasites have virulent or avirulent genotypes, are these fixed phenotypes independent of the gut microbiome or, more broadly, independent of the environment? An increasing number of studies suggest that the outcome of host/parasite interactions is not fixed by genetic factors. These studies address the role of exogenous or endogenous factors on the expression of both host and parasite genes during infection (Ferguson and Read, 2002; Thomas and Blanford, 2003; Barrett and Agrawal, 2004; Mitchell et al., 2005; Lambrechts et al., 2006; Salvaudon et al., 2007; Wolinska and King, 2009). If the gut microbiome is involved in host/parasite interactions including the role of the gut microbiome and its "meta-genome" (mG), this suggests that parasitism involves a G × G × mG interaction. Furthermore, if the gut microbiome is important to host/parasite outcomes, then the parasite must adapt to the demands of a dynamic molecular environment, e.g., the microbiome itself varies due to ontogenic development as well as physiological stresses (Koch and Schmid-Hempel, 2011; Benesh and Hafer, 2012). The gut microbiome- and/or context-dependent effects on the molecular cross-talk of host by parasite interactions could be described by the reaction norms (an inherited concept of genetics and basically applied to phenotypes, Woltereck, 1909), here it can be depicted, at least in part, as the variety of molecular patterns produced by a single G x G interaction across different gut microbiomes and/or contexts. This represents a higher complexity level compared to the G x G interactions that usually include two genetic changes in a single context or environment. Although the occurrence of such context or gut microbiome-dependent fluctuations is now assumed, their impact in altering the magnitude and the direction of the interaction has received little attention. Deeper knowledge of these complex interactions could provide a wealth of information for deciphering variability of the dynamics between host and parasite (**Figure 1**). Omics methodology provides an approach for efficiently detecting specific host or parasite molecular plasticity correlating with fluctuations in the gut microbiome. These methodologies also provide a gate to trace specific genes displaying broad adaptive value. Although this approach is limited to simple model systems because of its complexity, these methods could provide interesting clues to co-evolutionary processes. Moreover, the deciphering of these interactions will generate new hypotheses for the parasitic manipulation theory. The integration of the gut microbiome as a player involved in the process of the alteration of host behavior (Poulin, 2010) may even prove necessary for understanding host/parasite interactions.

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Cooperation and conflict in host manipulation: interactions among macro-parasites and micro-organisms

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Several parasite species are known to manipulate the phenotype of their hosts in ways that enhance their own transmission. Co-occurrence of manipulative parasites, belonging to the same species or to more than one species, in a single host has been regularly observed. Little is known, however, on interactions between co-occurring manipulative parasites with same or different transmission routes. Several models addressing this problem have provided predictions on how cooperation and conflict between parasites could emerge from multiple infections. Here, we review the empirical evidence in favor of the existence of synergistic or antagonistic interactions between co-occurring parasites, and highlight the neglected role of micro-organisms. We particularly discuss the actual importance of selective forces shaping the evolution of interactions between manipulative parasites in relation to parasite prevalence in natural populations, efficiency in manipulation, and type of transmission (i.e., horizontal versus vertical), and we emphasize the potential for future research.

Keywords: extended phenotype, horizontal transmission, host manipulation, multidimensionality, trophic transmission, vertical transmission

Several parasite species have evolved the ability to alter the phenotype of their hosts in ways that enhance their own fitness at the expense of that of their hosts (Moore, 2002; Thomas et al., 2005; Lefèvre et al., 2009). Such cases of so-called "host manipulation" by parasites are commonly regarded as compelling examples of extended phenotypes (Dawkins, 1982), and can be conveniently grouped in two broad categories depending on the way host fitness is affected. First, parasites may increase mortality risks faced by their host in order to increase their own transmission rate. For instance, increased horizontal transmission has been regularly evidenced in parasites with complex life cycles (such as acanthocephalans, cestodes, or trematodes) that alter the phenotype of their invertebrate intermediate hosts in ways that appear to increase vulnerability to predation by vertebrate final hosts (Bethel and Holmes, 1973; Kaldonski et al., 2007; but see below). Second, parasites may increase their transmission by altering the reproduction of their hosts in different ways. For instance, various vertically transmitted obligate intracellular parasites parasites, such as *Wolbachia* bacteria or microsporidia, have evolved the ability to feminize their hosts, thus increasing their rate of transmission (Terry et al., 2004; Werren et al., 2008; Engelstädter and Hurst, 2009) whereas helminths and over parasites may induce partial or total castration in their hosts (Thomas et al., 1996; Bollache et al., 2002; Jensen et al., 2006), resulting in the reallocation of host resources towards maintenance, and, hence, improved development and survival of the parasites.

Most studies of host manipulation by parasites have considered the case where hosts are infected by only one macroparasite, or a single parasite species (see Moore, 2002 for a review). However, multiple and/or mixed parasite infections have been shown to occur in a large range of host–parasite associations (Graham, 2008; Rigaud et al., 2010; Ferrari and Vavre, 2011; Viney and Graham, 2013), with the possibility that interactions between co-occurring parasites influence the timing and/or the type of phenotypic alterations observed in infected hosts. In particular, manipulative parasites may share a common interest in host manipulation or, alternatively, may compete to take the control of their common host (Lafferty, 1999), particularly when they depend on alternative pathways for successful transmission. So far, the potential for cooperation or conflict between co-occurring parasites has been largely addressed from a theoretical point of view, whereas empirical evidence remains limited. Here we review the literature on cooperation and conflict in host manipulation, discuss the importance of transmission routes and prevalence in shaping interactions between co-occurring parasites, and particularly emphasize the neglected role of micro-organisms.

HOST MANIPULATION: A CRITICAL INTRODUCTION

Before addressing the relevance of interactions between parasites to the study of host manipulation, it is worth examining the extent and variety of phenotypic alterations brought about by parasites in their hosts, as well as their consequences for both parties in the interaction. Depending on the host-parasite system under consideration, parasite-induced phenotypic alterations can consist of modified appearance, aberrant behavior, disrupted physiology, or a combination of the three (Moore, 2002; Cézilly et al., 2013). As an illustration, we will focus here on hosts infected with either helminths with complex life cycle or microorganisms. A more complete description of the effects of other types of parasites on the phenotype of their hosts can be found in Moore (2002) and Poulin (2007).

PHENOTYPIC ALTERATIONS CAUSED BY HELMINTHS WITH COMPLEX LIFE CYCLE IN THEIR INTERMEDIATE HOSTS

Many helminth species with a complex life cycle can alter the phenotype of their hosts in different ways. First, they can modify their appearance, with the effect of making them more conspicuous. One famous example corresponds to bird trematodes belonging to the genus *Leucochloridium* infecting snails as intermediate hosts. Transmission of the parasite to its definitive avian hosts is supposedly facilitated by the rhythmic movement of colored sporocyst broodsacs in the ocular tentacles of infected snails. The resemblance of the broodsacs to caterpillars is believed to lure birds and, thus, increase the probability of trophic transmission to the final host (Lewis, 1977). Second, parasites may affect the behavior of their hosts. For instance, snails infected with *Leucochloridium* have been reported to become positively phototactic and to position themselves in places, situated higher in the vegetation, what presumably increases their exposure to bird predators (Wesołowska and Wesołowski, 2014). However, direct evidence for differential predation of birds on infected snails is lacking.

Interestingly, this typical text-book example of host manipulation comes as a cautionary tale, as it perfectly illustrates two important aspects of the study of host manipulation. First, as observed in snails infected with *Leucochloridium*, infection with manipulative parasites most often affects more than one dimension in host phenotypes, even though earlier studies have generally considered the influence of infection on a single trait at a time. Indeed, although multidimensionality in host manipulation has received attention only recently (Cézilly and Perrot-Minnot, 2005, 2010; Thomas et al., 2010), it seems to be the rule rather than the exception (Cézilly et al., 2013). Second, most studies have concluded that manipulative parasites with complex life cycle benefit from inducing phenotypic changes in their hosts in terms of increased trophic transmission without necessarily proving it. The mere observation that parasite-induced phenotypic alterations show "signs of purposive design" (Poulin, 1995) and are associated with increased susceptibility of infected intermediate hosts to predation by final host is actually no evidence for a causal relationship between the two phenomena (Cézilly et al., 2010), contrary to what has been commonly inferred in the study of host manipulation by parasites, as shown by studies of acanthocephalan parasites and their amphipod hosts.

Amphipod species (crustaceans) are regularly exploited as intermediate hosts by fish and bird acanthocephalans. In several of them, mature cystacanths (the infective stage to the definitive host) show typical carotenoid-based orange colorations (see Gaillard et al., 2004) which are visible through the translucid cuticle of their crustacean intermediate hosts, and, presumably, make them more conspicuous to predators. In addition, infection with such parasites generally alters the natural negative phototaxis of their hosts, such that infected individuals become indifferent or attracted to light, whereas uninfected ones are strongly repulsed by it (Bethel and Holmes, 1973; Cézilly et al., 2000). However, the phenotypic effect of infection with acanthocephalans goes well beyond altering appearance and reaction to light as, for instance, no less than 15 different phenotypic alterations have been reported in the crustacean amphipod *Gammarus pulex* infected by the acanthocephalan parasite *Pomphorhynchus laevis*, including several physiological effects such as reduced oxygen consumption, decreased immunocompetence, increased brain serotonergic activity, and increased glycogen levels (Cézilly et al., 2013). Besides, predation experiments have shown that amphipods infected by fish acanthocephalans are more vulnerable to fish predation than uninfected ones (Bakker et al., 1997; Kaldonski et al., 2007), suggesting that at least some of the phenotypic alterations observed in infected hosts might be adaptive for the parasite. Accordingly, following a series of experiments, Bakker et al. (1997) concluded that both altered host appearance and phototactic behavior were responsible for the increased vulnerability of *G. pulex* infected with the fish acanthocephalan *Pomphorhynchus laevis* to predation by fish, thus providing supposedly firm evidence for the host-manipulation hypothesis. However, more recent studies have shown that this conclusion was erroneous. Using realistic painted mimics, Kaldonski et al. (2009) showed that cystacanths color actually plays no causal role in the increased susceptibility of infected amphipods to fish predation. And nor does altered phototaxis, as evidenced by Perrot-Minnot et al. (2012), through resorting to both phenotypic engineering and predation experiments under contrasted light intensities. One possibility, that remains so far untested, is that the increased vulnerability of infected hosts is due to a combination of phenotypic alterations rather than a single one, hence the importance of addressing multidimensionality in manipulation. Alternatively, infected amphipods might simply be less vigorous than uninfected ones and, hence, less able to escape from predators (Cézilly et al., 2010). Whatever the correct answer is, the investigation of the adaptive consequences of phenotypic alterations induced by parasites with trophic transmission clearly deserves further development.

Helminths with complex life-cycles are also known to alter the reproductive biology of their hosts through reducing male competitiveness and inclination to pair (Zohar and Holmes, 1998; Bollache et al., 2001), or through decreasing female fecundity through partial or total castration (Poulton and Thompson, 1987; Bollache et al., 2002). The impact of helminth parasites on the reproductive physiology of their intermediate hosts seems to be a direct consequence of the energetic cost of parasitism. For instance, in the freshwater isopod intermediate host *Ceacidotea communis* infected by the acanthocephalan parasite *Acanthocephalus tehlequahensis*, infected individuals have been found to allocate about 21% of their net production energy to parasite growth, while allocating zero energy to reproduction (Lettini and Sukhdeo, 2010).

So far, to the best of our knowledge, the effect of helminth parasites on the reproductive biology of their intermediate hosts has been investigated independently of their manipulative effects on other phenotypic dimensions. An important question, however, in the study of host manipulation by parasites is whether those different alterations are independent of each other from a mechanistic point of view, or are linked by common physiological mechanisms of dysfunction (Cézilly and Perrot-Minnot, 2010).

MICRO-ORGANISMS ASSOCIATED WITH HOST PHENOTYPIC CHANGES

A large variety of micro-organisms, ranging from viruses to protozoa, has been shown to modify their host phenotype, and these phenomena have often been interpreted as cases of host manipulation. Two broad categories can be distinguished. The first involves microparasites modifying host reproduction, for which numerous reviews are available (O'Neill et al., 1997; Duron et al., 2008; Engelstädter and Hurst, 2009). A now classical example of such microorganisms are *Wolbachia* bacteria (O'Neill et al., 1997; Werren et al., 2008), but recent studies evidenced a large spectrum of microorganisms inducing similar effects, probably showing evolutionary convergence (e.g., Terry et al., 2004; Duron et al., 2008; Ferrari and Vavre, 2011) These micro-organisms all show a common trait: they are vertically transmitted, mostly *trans*-ovarially. They may increase the proportion of their transmitting female hosts through inducing sex-reversal in males, or parthenogenesis, or male-killing (Bandi et al., 2001). They may also prevent uninfected individuals or individuals infected with a different strain from reproducing, through inducing cytoplasmic incompatibility (Stouthamer et al., 1999; Perrot-Minnot et al., 1996). Such alterations of host reproductive biology appear to be obligatory for the maintenance of these micro-organisms inside their host, and often favor their spread and fixation. In these cases, the adaptive nature of these reproductive changes for parasite transmission is doubtless.

The second category of manipulative microparasites is those inducing changes in host behavior, as those evidenced for several macro-parasites (see above). There is a growing body of evidence for these changes, encompassing parasites with complex life-cycle and either trophic or vector-borne transmission, and parasites with direct life-cycle (reviews in Lefèvre et al., 2006, 2009; Ezenwa et al., 2012; van Houte et al., 2013). Most of them, if not all, have been interpreted as changes in the host behavior favoring parasite transmission to next hosts, as illustrated in the following examples. In the heterogenous parasite *Toxoplasma gondii*, infected rats become attracted by cat scents, favoring the parasite trophic transmission after ingestion by a feline definitive host (Berdoy et al., 2000; Vyas et al., 2007; but see Worth et al., 2013). Several vector-borne protozoans alter the probing rate, probability of multiple feeding and host choice of their dipteran vector, in ways increasing their chances of getting transmitted (Koella et al., 1998; Lefèvre et al., 2006; Cator et al., 2012). Interestingly, the stage-specific changes in host seeking and attraction induced by *Plasmodium yoelii* infection in its vector *Anopheles stephensi* can be mimicked in uninfected mosquitoes by an immune challenge (Cator et al., 2013). Among vertically transmitted parasites, the filamentous virus (LbFV) infecting the parasitoid *Leptopilina boulardi* is responsible for superparasitism behavior in its female host (Varaldi et al., 2003). Instead of laying their egg in an uninfected *Drosophila* larvae (an optimal laying behavior avoiding superparasitism in this species), *L. boulardi* females infected by LbFV parasitize *Drosophila* larvae already hosting another *Leptopilina* larvae. Experimental data and theoretical modeling showed that by doing so, infected *L. boulardi* mother increase the probability of virus transmission: during intra-*Drosophila* competition, larvae infected by vertical transmission often transmit horizontally the LbFV to other *Leptopilina* larvae, increasing the probability of parasite maintenance in host populations (Varaldi et al., 2003; Gandon et al., 2006). Finally, various insect species, when infected by fungi or viruses with direct life-cycle, exhibit abnormal climbing and clinging behavior in the hours preceding their death. The infectious stages of these parasites are released from dead hosts, and the position of hosts above ground or at the top of plants could increase the chances of dissemination (Goulson, 1997; Roy et al., 2006; Andersen et al., 2009).

In addition, recent studies have evidenced a "puppet master" role of microorganisms in what was thought to be alterations in host behavior brought about by macroparasites. Some ladybird species, when parasitized by Braconidae parasitoids, appear to protect the parasitoid pupae from entomophagous predators through adopting a modified behavior (Maure et al., 2013). It has been recently shown in one of these cases that the "body-guard" behavior expressed by "zombie" hosts is actually induced by a virus released by the developing parasitoid larvae inside the host (Dheilly et al., submitted). As in a system of Russian dolls, the vertically transmitted virus seems to favor its own transmission through increasing the survival of its parasitoid host thanks to the protective behavior it induces in the parasitoid's host. Another form of protective manipulation possibly involving a symbiont of the parasite is aposematism. The entomopathogenic nematode parasite *Heterorhabditis* enhances its survival by inducing in its dead larval moth the production of warning colorations and distasteful chemicals host that deter bird predators (Fenton et al., 2011). The suspected role of *Photorhabdus luminescens*, the mutualist bacterium of the nematode and the actual parasite of the larval moth, in these changes remains to be demonstrated. Such cases of "ménage à trios" illustrate the importance of considering microorganisms as key players in parasitic interactions, with important consequences on the evolution of virulence itself.

It is worth noting, however, that changes in behavior following infection with a microparasite may sometime correspond to a strategy by which hosts eliminate the infection or reduce its costs, as shown by changes in food preferences (leading to self-medication) or behavioral fevers (insects seeking high temperatures unfavorable to parasite growth) (see Perrot-Minnot and Cézilly, 2009, for a review).

INTERACTIONS BETWEEN CO-OCCURING PARASITES: THEORETICAL CONSIDERATIONS

Most parasites have highly aggregated spatial distributions (Combes, 1991), meaning that parasites of the same species tend to co-occur within a single host more often than by chance. As a consequence, monospecific mixed infections involving at least two strains are common in nature (several references in Read and Taylor, 2001; Choisy and de Roode, 2010; Alizon et al., 2013). They can give rise to intraspecific competitive or cooperative interactions over host exploitation, with important consequences for the evolution of virulence (Read and Taylor, 2001; Buckling and Brockhurst, 2008; Lively, 2009; Choisy and de Roode, 2010; Alizon et al., 2013). In addition, interactions between co-occurring

parasites might be mediated by the host immune system (see for instance Ulrich and Schmid-Hempel, 2012; Fairlie-Clarke et al., 2013). However, because little is known about host-mediated competition between manipulative parasites, we will only consider here direct parasite-parasite interactions, although the role of host immunity clearly deserves further attention. In the case of manipulative parasites, co-infection may promote the evolution of cooperation in manipulation at the within-host level if transmission interests are closely aligned among conspecifics. In this case however, conflict or cooperation may still arise on the distribution of metabolic costs of manipulation among co-infecting parasites, i.e., on the relative manipulative effort. In any case, intraspecific interactions should have direct consequences on the individual optimal level and pattern of manipulative effort. As evidenced for parasite establishment, growth, and reproduction, several parameters are expected to impact the individual parasite's decision on manipulative strategy in a co-infected host, such as relatedness, parasite load, timing of co-infection and host density (i.e., opportunities for transmission). In addition, parasites belonging to different species, and having similar or contrasted transmission routes, can coexist in a single host. Multiple infections, i.e., individual hosts co-infected by different parasite species, are frequent in nature (Rigaud et al., 2010). They have received recent theoretical attention because of their potential effect on the evolution of parasite virulence and/or transmission (Alizon et al., 2013). In the case of manipulative parasites, co-infection may occur between individuals sharing a common interest or opposite ones. Under the assumption that manipulation is costly, the co-occurrence of several parasites within a single host may then lead to synergistic or antagonistic interactions (Brown, 1999; Lafferty, 1999; Vickery and Poulin, 2010).

The simplest situation is when two or more manipulative parasites belong to a single species and, hence, sharing a common transmission strategy, co-occur in the same host. Brown (1999) modeled a situation where the fitness of each individual parasite decreases as its manipulative effort increases (reflecting the cost of manipulation), and increases as a function of the total manipulation effort achieved by all parasites present in the host. Under such circumstances, each individual parasite may lower its own manipulative effort while keeping constant its probability of transmission or, even, increasing it (Brown, 1999; Vickery and Poulin, 2010). However, the extent of cooperation between parasites is highly dependent upon both n, the size of the infrapopulation (being zero when n is small), and the efficiency of "passive" transmission (i.e., transmission when no manipulation occurs). Besides, an individual's investment in host's behavioral manipulation will benefit others in terms of increased transmission chances to the next host, and, as such, manipulative effort directed towards increased transmission success is a form of public good. Such strategy might therefore be strongly sensitive to relatedness among co-infecting parasites (see Buckling and Brockhurst, 2008; Lively, 2009, for a discussion on how relatedness affect optimal host exploitation), and to whether costs and benefits from manipulation are equally distributed among coinfecting strains. Under low relatedness, the presence of other strains within a host is expected to lower the per-strain investment in host manipulation because parasites should be less willing to help non-kin (Brown, 1999; Vickery and Poulin, 2010), because of a trade-off in resource allocation under high within-host competition, or because cheating mutants that save on the metabolic cost of manipulation have a selective advantage when co-infecting with manipulative strains (Buckling and Brockhurst, 2008). The first two cases involve a plastic response to co-infection on manipulative effort, whereas the latter one involves an evolutionary response.

A different situation occurs in heterospecific co-infections, when one parasite species which is unable to manipulate the phenotype of its intermediate host's phenotype benefits from the manipulative effort of another parasite species, with which it shares a common final host. Thomas et al. (1997) coined the term "hitchhiking" to account for the possibility that a non-manipulative species develops an ability to differentially infest hosts already infected by a manipulative one. To our knowledge, the evolutionary dynamics of such a situation has not been investigated so far. One prediction, however, is that hitchhiking should result in a non-random association between the manipulative species and the hitchhiking one among hosts.

A potential for conflict between co-occurring parasites can exist under different circumstances. First, parasites of the same species may have different interests in transmission in terms of timing. It has long been theoretically predicted and experimentally demonstrated that adaptive manipulation enhances transmission specifically when the developmental stage infective to the next host has been reached by the parasite (Parker et al., 2009: manipulation by "predation enhancement"). More recently, the selective advantage of manipulating the host in ways protecting the parasite from premature transmission until infective to the next host has been acknowledged (manipulation in the form of "predation suppression," Parker et al., 2009). It has thus been predicted that opposite interests between developmental stages should translate into conflict over host manipulation when sequential infection with conspecific parasites occurs (Parker et al., 2009). Given that non-specific predation suppression may evolve more easily than predation enhancement (Parker et al., 2009), and that non-infective stages have more to lose from premature trophic transmission (dead-end) in co-infection than the manipulative infectious stage has from delayed manipulation (time), non-infectious stage are expected to win the conflict over the timing of manipulation.

Second, conflict may occur between trophically transmitted parasites that rely on different definitive hosts to complete their life cycles, and, hence, are not compatible, when at least one of the two is a manipulative parasite. In such a case, different outcomes can be predicted. Conflict of interest may lead parasites species to avoid hosts already infected by a non-compatible parasite species (Lafferty, 1999). This would result in a lower rate of co-infections between non-compatible parasites than expected by chance. Alternatively, one parasite species may out-compete the other and manage to impose its own interest in transmission. For instance, a non-manipulative parasite might be able to prevent its host from being manipulated by

a non-compatible parasite, such that co-infected host resume to normal phenotype. In the case of a conflict between two non-compatible, manipulative parasites, the host would express the altered phenotype induced by the most competitive parasite. Finally, non-compatible manipulative parasites might be unable to counteract each other, resulting in mixed altered phenotype in the host.

Third, conflict may oppose parasites with horizontal transmission to parasites with vertical one, when the former has a negative impact on the reproductive output of their common host. Total or partial castration of the intermediate host by a manipulative macroparasite would clearly be detrimental to the fitness of vertically transmitted microparasites, such that natural selection may have favored in the latest the ability to counteract castration, fully or partially.

INTERACTIONS BETWEEN CO-OCCURING PARASITES: EMPIRICAL EVIDENCE

EVIDENCE FOR COOPERATION BETWEEN CONSPECIFIC PARASITES INFECTING A SINGLE HOST

Only a few studies so far have examined in detail the effect of multiple infections by a single parasite species on host manipulation. Using naturally infected *G. pulex*, Cézilly et al. (2000) observed no change in altered behavior with the intensity of infection with either *Pomphorhynchus laevis* or the bird acanthocephalan *Polymorphus minutus*. Conversely, using experimental infections of *G. pulex* by *Pomphorhynchus laevis*, Franceschi et al. (2008) found that manipulation (reversed phototaxis) was higher in hosts infected with two parasites than in singly infected ones, with no further increase in manipulation at higher intensities. In addition, using the same host-parasite association, Dianne et al. (2012) observed that all co-infecting parasites did not equally suffer from intraspecific competition. Larval size was positively correlated with host phototaxis in single-infected individuals, but not at higher infection intensities, possibly because competition for host resources affects larval growth and manipulative abilities of co-infecting larval acanthocephalans.

Host-parasite associations where the investment into manipulation is associated with a specific localization in the host provide better opportunities to evaluate the potential for sysnergistic interactions at the intraspecific level. For instance, metacercariae of the trematode *Curtuteria australis* (Echinostomatidae) all encyst in the foot muscle of their host, the New Zealand cockle, *Austrovenus stutchburyi,* but only those localized at the tip of the foot can impair borrowing behavior, hence increasing trophic transmission to avian final hosts. This manipulative strategy comes at a cost since fish cropping of the foot tip decreases the chances of survival before getting transmitted of individuals encysted there (Leung et al., 2010). Surprisingly, newly arriving cercariae encyst in the foot tip of already infected cockles, instead of taking the opportunity of a safer location without loosing the benefits from impaired burrowing on transmission success. This apparent cooperation in manipulative effort may arise due to the necessity to reach a minimum threshold number of cercariae to significantly impair burrowing (Leung et al., 2010).

CONFLICT BETWEEN DEVELOPMENTAL STAGES OVER THE TIMING OF TRANSMISSION

Several studies of the phenotypic alterations brought about by trophically transmitted parasites have provided evidence for a switch in the behavior of infected intermediate hosts from reduced exposition to predation to increased one over the course of the parasite's development (Hammerschmidt et al., 2009; Dianne et al., 2011; Weinreich et al., 2013). A similar phenomenon has been reported in vector-born malaria parasites, with opposite effects of the infectious stage (sporozoïte) and the pre-infectious stage (oocyst) on the behavior of mosquitoes, more specifically on blood-feeding and other risky behaviors (several references in Cator et al., 2012, 2013).

However, to our knowledge, only two studies have examined the evidence in favor of a conflict over the timing of transmission. Sparkes et al. (2004) examined the potential for such a conflict in the acanthocephalan parasite *Acanthocephalus dirus* that is known to induce a color change, from dark to light-colored, in its intermediate host, the aquatic isopod *Caecidotea intermedius*, supposed to increase exposure to predation by fish final hosts (Camp and Huizinga, 1979). Non-infective stages (acanthella) of the parasite induce a color change over about 40% of the host's body, whereas infective ones induce a color change above 80%. Despite a potential for conflict over the extent of color change, Sparkes et al. (2004) found that hosts co-infected with infective and non-infective stages were similar in the degree of color change to hosts infected by an infective stage, contrary to theoretical predictions (Parker et al., 2009). On the other hand, using both field data and experimental infections, Dianne et al. (2010) have shown that the presence *Pomphorhynchus laevis* acanthella delays the reversal of phototaxis induced by cystacanths (mature parasites), without however suppressing it, resulting in an intermediate level of manipulation when co-infection associates the two larval stages.

CO-INFECTIONS WITH HETEROSPECIFIC PARASITES

Multiple infections between heterospecific parasites are commonly observed in the wild, and, in some cases, occur more often than by chance, possibly because variation exists between hosts in immunocomptence and, hence, susceptibility to infection (Poulin, 2007). However, detailed studies on interspecific interactions involving at least one manipulative parasite are few.

In particular, evidence for hitch-hiking between a non-manipulative parasite and a manipulative one remains limited. According to Thomas et al. (1997), the trematode *Maritrema subdolum* increases its own transmission to avian final hosts through preferentially infecting *Gammarus insensibilis* already infected by the trematode *Microphallus papillorobustus*. The latter appears to enhance its transmission to aquatic birds by inducing in their amphipod hosts a positive phototaxis, a negative geotaxis and an aberrant evasive behavior, which presumably make them more susceptible to predation by aquatic birds (Helluy, 1984). Metacercariae of *M. subdolum* were positively associated with those of *M. papillorobustus* among amphipod hosts in the field. In addition, laboratory experiments showed that cercariae of *M. subdolum* actively swam towards the top of the water column, then increasing their probability of encountering amphipods already infected

by *M. papillorobustus*. However, Mouritsen (2001) contended that cercariae of *M. subdolum* actually do not swim in the water column, but instead crawl at the sediment–water interface, and concluded that *M. subdolum* cannot be a hitch-hiker as suggested by Thomas et al. (1997), whereas a likely candidate would be another species of *Microphallus*. Altenatively, the difference between the two studies might be due to the fact that the behavior of the cercariae of *M. subdolum* is different in different regions (Thomas and Helluy, 2002), although the reason for such a difference remains obscure.

A similar case of non-random association between a non-manipulative parasite and a manipulative one has been investigated by Leung and Poulin (2007a,b). It consists in a positive association between infection intensity of the metacercariae of foot-encysting echinostomes and that of gymnophallid metacercariae in their common host, the cockle, *Austrovenus stutchburyi*. The authors first suggested that the gymnophallid was a hitch-hiker parasite because, in addition to the pattern of positive association, it shares the same transmission route as the echinostomes, but unlike the echinostomes, it is unable to manipulate the burrowing behavior of cockles, and increase its transmission to avian final hosts (Leung and Poulin, 2007a). To test this hypothesis, they conducted a field experiment involving cockles forced to remain either above or below the sediment surface to simulate manipulated and non-manipulated hosts. There was however no evidence for a preference of gymnophallids for either surfaced or buried cockles, thus refuting the hitchiking hypothesis (Leung and Poulin, 2007b).

Evidence for synergistic efforts in manipulation between different parasite species remains scarce, too. Poulin et al. (2003) investigated interactions among three helminth species, one acanthocephalan, one trematode and one nematode, co-occurring in two species of crab as intermediate hosts and exploiting shorebirds as final hosts. They found no measurable effect of other helminth species on the size of acanthocephalans, suggesting no interspecific conflict over resource use within crabs. However, concentrations of serotonin (a neuromodulator involved in the altered phototaxis of crustaceans hosts infected with acanthocephalans and trematodes, Helluy and Thomas, 2003; Tain et al., 2006, 2007) in the brains of one crab species were negatively related to the numbers of acanthocephalans and trematodes, but not to nematodes, suggesting of a potentially synergistic manipulation of host behavior by the two helminth species.

Although conflict in manipulation seems obvious when manipulative parasites targeting contrasted species as final hosts co-occur in a single intermediate host, only a few studies have directly assessed the outcome of such interactions. Cézilly et al. (2000) examined the alteration of both phototaxis and geotaxis in *G. pulex* co-infected by *Pomphorhynchus laevis* and *Polymorphus minutus* and found that outcome of the antagonism between the two parasites differed between the two traits. One the one hand, reversed geotaxis (inducing hosts to swim closer to the surface where they presumably become more exposed to bird predators) of gammarids harboring both parasites was less pronounced than that of individuals infected with only *Polymorphus minutus*. On the other hand, altered phototaxis of individuals infected with both parasites was similar to that of individuals infected with only *Pomphorhynchus laevis*. In addition, using large samples collected in the field, Outreman et al. (2002) found no evidence for non-random association between the same two parasite species among their common intermediate hosts.

Looking at interactions between more distantly related parasite species may, however, provide a different perspective. For instance, using naturally infected individuals, Rauque et al. (2011) found that co-infection with the trematode *Microphallus* sp. impaired the ability of both the acanthocephalan *Acanthocephalus galaxii* and a cyclophyllidan cestode to alter the phototaxis of the amphipod *Paracalliope fluviatilis*. However, caution must be exerted when interpreting such results. For instance, Fauchier and Thomas (2001) found a negative association between the non-manipulative nematode *Gammarinema gammari* and *M. papillorobustus* among male *G. insensibilis* in the field, suggestive of a conflict of interest between the two parasites. Indeed, Thomas et al. (2002) showed that among amphipods naturally infected by the trematode, those who did not display altered phototaxis also had more nematodes, suggesting that the nematode could cancel the manipulation effort of the trematode, and hence avoid a premature death. However, altered phototaxis was maintained after exposing manipulated amphipods to nematodes, while the experimental elimination of nematodes from non-manipulated co-infected amphipods did not restore manipulation.

HORIZONTAL TRANSMISSION, VIRULENCE AND CASTRATION VS. VERTICAL TRANSMISSION

Among multiple parasite infections, one case deserves particular attention because of its strong potential source of conflict: the co-infection by manipulative parasites and vertically transmitted (VT) parasites. There is now growing evidence that VT, maternally transmitted, microorganisms are ubiquitous in invertebrates (Vautrin and Vavre, 2009). In all cases, vertical transmission necessitates survival of the "vector" host, i.e., the mother. As noted earlier, most manipulative parasites increase host mortality. Trophically transmitted parasites are of particular interest because, as for parasitoids, their transmission critically relies on host death. Indeed, their transmission to the definitive host implies the death of the intermediate host by predation, a phenomenon that can be seen as an extreme example of virulence (Poulin and Combes, 1999). VT parasites or symbionts often reach high prevalence in hosts' populations. At the individual level, they are present in a given host individual at its birth, i.e., before any other super-infection by a horizontally-transmitted (HT) parasite. Therefore, provided the risk of infection by a virulent HT parasite is not too low, natural selection should potentially favors VT parasite variants able to fight either the presence or the effects of HT virulent parasites (Rigaud and Haine, 2005).

Several studies are now available showing that such symbiont-mediated protection (Haine, 2008) have been selected against parasitoids or other types of parasites lethal to the host (e.g., Rouchet and Vorburger, 2012; Lukasik et al., 2013, respectively, for recent examples). Models on the ecology and evolution of this type of conflict between VT and HT parasites revealed that protection is more likely to evolve (i) in long-living hosts, (ii) in

response to HT that causes a significant reduction in host fecundity (e.g., those castrating their hosts), but inducing moderate levels of virulence (Jones et al., 2011). However, protective symbionts are typically found in only a fraction of the host population, a phenomenon that could be due to a very fine balance between the costs induced by symbionts and the strength of protection they provide (Kwiatkowski and Vorburger, 2012).

Only a few studies testing specifically the conflicts between VT and manipulative parasites are available. One study tested the effect of a VT, feminizing, microsporidium (*Dictyocoela* sp.) on the outcome of co-infections with acanthocephalans in their *G. roeseli* hosts (Haine et al., 2005). The VT parasites did not protect their hosts against acanthocephalan infections, but reduces the geotaxis inversion induced by *Polymorphus minutus* (a behavioral change increasing the probability of predation by the bird definitive host). This "sabotage" of behavioral change occurs against the acanthocephalan species that, in addition, castrate 75% of female hosts. Sabotage was not found against *Pomphorhynchus laevis*, another acanthocephalan species inducing a moderate reduction in fecundity (Haine et al., 2005), fitting the prediction of Jones et al. (2011) that VT parasites could more easily select for resistance against a castrating parasite. However, the microsporidia did not influence the degree of castration among females infected by *Polymorphus minutus.* A second study explored the effect of the partial VT microsporidian parasite *Octosporea bayeri* (also using horizontal transmission in its life-cycle) when occurring in co-infections with the HT blood-infecting, castrating, bacterium *Pasteuria ramosa* in *Daphnia* hosts (Ben-Ami et al., 2011). When the two parasites co-infect the hosts after both using horizontal transmission, *Pasteuria* competitively excluded *Octosporea*, and characteristics of double infections resembled those of single infection by *Pasteuria.* When hosts became first vertically (transovarilly) infected with *Octosporea*, there was no evidence that the VT parasite protects its host against *Pasteuria*, neither in protecting from co-infection nor in avoiding castration. However, *Octosporea* was able to withstand competition with *Pasteuria* to some degree, and was able to produce infective stages. However, both parasite species suffer from the co-infections (i.e., produced less infective stages than in single infections) and co-infections led to the expression of higher virulence, a probable consequence of intra-host parasite competition.

These two examples differ in many characteristics of both hosts and parasites life-histories. The differences in the outcome of multiple infections between VT and HT parasites are therefore not surprising. However, it is worth noting that acanthocephalan/microsporidia multiple infections in *Gammarus roeseli* are not rare in the wild (see also Gismondi et al., 2012), while *Pasteuria*/*Octospora* multiple infections in *Daphnia* are much rarer in natural populations (Ebert et al., 2001). The differences in strength of the selective pressure exerted by the HT parasites, i.e., in the prevalence of multiple infections, might therefore explain why in one case host protection by "sabotage" has been selected in VT parasites and not in the other example.

CONCLUSION AND PERSPECTIVES

Although, theoretical considerations suggest that interactions between parasites within a single host may shape manipulation, the available empirical evidence, at least concerning interactions between macroparasites in invertebrate hosts, is surprisingly thin. This may be due to several reasons. First, although appealing, predictions about a plastic adjustment or an evolutionary response of individual manipulative effort to co-infection are difficult to test experimentally. Indeed, in most host-parasite systems, individual manipulative effort can be only indirectly estimated from its consequence on the parasite's fitness in the next host, such that potential trade-offs between manipulation of intermediate host and survival and reproductive success within the final host are most often difficult to quantify. Second, competition rather than cooperation might the dominant force in infra-populations of manipulative parasites, if, for instance, parasites that are co-occurring in a single intermediate host suffer from reduced size and fecundity (see for instance Fredensborg and Poulin, 2005). Third, the potential conflict of interest between co-occurring manipulative parasites might be reduced if there is a high risk of dying within the intermediate host before reaching a definitive host. In such a case, reducing such a risk at the expenses of specificity in transmission may be favored by natural selection (see Seppälä and Jokela, 2008; Cézilly et al., 2010). Fourth, it has been suggested that given the relatively low frequency of manipulative parasites, co-occurrence is generally a very rare event in the field. Consequently, such a weak selection pressure should have no important consequences beyond the host individual level (Rauque et al., 2011). However, this might not be a correct way of reasoning. Let's consider the hypothetical case of two manipulative parasite species, A and B, exploiting in sympatry a common intermediate host but depending on different species as final hosts, and, hence, inducing contrasted phenotypic alterations. Let's assume that the prevalence of parasite A is 5%, whereas that of parasite B is 20%. In the absence of non-random association, the expected prevalence of mixed infections among intermediate hosts would be as low as 1%. Still, any parasite A would still have a 20% chance of infesting a host already infected with parasite B, a rate that might be sufficient to favor the evolution of an ability for parasite A to overpower parasite B. Note that the symmetrical selective pressure would be less for parasite B, as its chance of co-occurring with parasite A when infecting an intermediate host is only 5%. This simple example suggests that the outcome of interactions between parasites may also depend on their relative prevalence within the intermediate host population.

Clearly, the study of the consequences of both monospecific and plurispecific co-infections by parasites on host phenotype deserves further consideration, both on theoretical and empirical grounds. In particular, more attention should be given about the importance of relatedness between parasites on the outcome of co-occurrence within a single host (Brown, 1999; see Keeney et al., 2007). In that respect, recent progress with experimental infections combined with the use of refined molecular tools to assess genetic relatedness may prove useful in the future.

However, we would like to emphasize here the neglected importance of micro-organisms in the study of host manipulation by parasites. Clearly, potential conflict between vertically transmitted micro-organisms and macro-parasites inducing total or partial castration offers interesting perspectives for future research. More generally, the relevance of microorganisms for the study of host

manipulation by parasites deserves further attention. Microorganisms are ubiquitous and can have important, although subtle effects on their hosts' phenotype. For instance, it is now accepted that variation in the composition of several microbiomes (gut or skin microbiota for example) can alter the behavior of various species (fruitflies, mosquitoes, or mice; reviewed in Ezenwa et al., 2012). These microbiomes are ubiquitous in animals, and almost nothing is known on their interaction with other "passengers" of their hosts, in particular manipulative parasites. Such interactions could be direct if the microbiomes influence the manipulative effects of parasites (possibly through the behavioral changes they induce themselves), or indirect, through the regulation of the host's immune system. For instance, it has been recently shown that non-pathogenic aquatic bacteria can activate the immune system and increase predation risk of *Enallagma cyathigerum* damselfly larvae (Janssens and Stoks, 2014). To what extent this result extends to other invertebrate species, and particularly to species that are exploited as intermediate hosts by macro-parasites with complex life cycle, remains to be assessed. So far, studies of the phenotypic alterations induced by macroparasites have not considered the possibility that hosts would be simultaneously infected by microorganisms, although this is very likely when studying naturally infected hosts collected in the field. Because of their reduced immunocompetence, hosts infected with certain micro-organisms, pathogenic or non-pathogenic, might be more likely to become infected by macroparasites (Cornet et al., 2009), opening the possibility that the observed, and often non-specific (see Kaldonski et al., 2008), increased vulnerability to predators of intermediate hosts infected with manipulative macro-parasites is a more complex phenomenon than previously thought. How much speculative such considerations can be, they strongly argue in favor of an increased interest in the role of micro-organisms in the phenomenon of host manipulation by parasites.

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