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Miriam Reverter

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CRIOBE USR 3278

Host-parasite interactions in coral reef fish

Par Miriam REVERTER

Thèse de doctorat de Biodiversité, génétique et évolution

Sous la direction de M. David LECCHINI, Directeur d'études EPHE

Soutenue le 21 Octobre 2016

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Have the courage to follow your heart and intuition. They somehow know what you truly want to become.

Steve Jobs

To my family and loved ones,

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General introduction

General introduction

Ecosystems are dynamic systems composed by the biological community and the abiotic environment. Organisms from the same ecological communities interact directly and indirectly forming the basis for many ecosystem properties and processes such as nutrient cycling and food webs (Agrawal et al. 2007). Organisms' interactions play an important role in the maintenance of biodiversity, mediation of ecosystem responses to global changes and ecosystem stability and resilience (Bascompte et al. 2006; Ives and Carpenter, 2007; Suttle et al. 2007; Tylianakis et al. 2008).

At the coarsest level, ecological interactions can be defined as intra-specific interactions, when they occur between individuals of the same species, or inter-specific interactions, occurring between different species (Fig. 1). However, since these interactions occur within ecological communities they can be affected and indirectly influenced by other species and their interactions (Land et al. 2015). Some of the most studied ecological interactions include competition, predation, symbiosis, commensalism and parasitism.

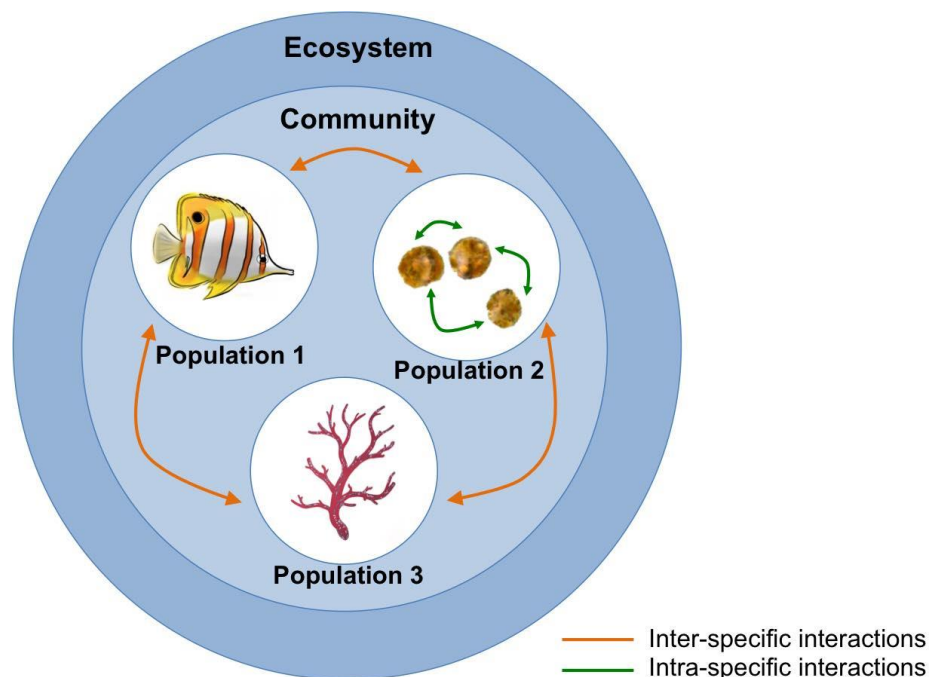


Figure 1. Organisms' interactions within a biological community.

1. Parasitism

Parasitism is a non-mutual relationship between species, where one species, the parasite, lives at the expense of the other, the host, causing deleterious effects (Combes, 2001). Parasites are ubiquitous, occurring in all food webs at all trophic levels and parasitism is considered to be the most common lifestyle on earth (Price, 1980; Lafferty et al. 2008a). Parasites are often classified depending their life cycle and their habitat within the hosts. A direct parasite has only one host, while an indirect parasite has multiple hosts, with one or more intermediate hosts and a definitive host. Parasites that live on the outer surfaces of the host like skin or gills are called ectoparasites, while those living inside the host are called endoparasites. There are two types of endoparasites, intercellular parasites like some worms, which inhabit spaces in the host's body and intracellular parasites such as protozoa, bacteria or viruses, that inhabit host cells. Transmission of ectoparasites, such as in monogenean worms, happens through direct contact between hosts. Endoparasites can infect their hosts either by penetrating its external surface or through parasite ingestion.

In host-parasite interactions, host populations develop defence mechanisms to avoid parasite infections, and parasites evolve counter-adaptations to overcome host defence mechanisms, which results in a tight host-parasite co-evolution (Klein et al. 2004). According to the Red Queen Hypothesis, hosts are at disadvantage during the course of co-evolution with their parasites because they tend to have longer generation times and smaller population sizes than their parasites (Kaltz and Shykoff, 1998).

If the host population is homogeneous and remains constant over time, evolution is expected to favor parasites that are best adapted for this host, what is known as parasite specialisation¹. In more rapidly changing or spatially heterogeneous host populations, parasites might find themselves exposed to a wider range of hosts, and therefore parasites tend to be generalists², favouring the highest geometric mean fitness across different hosts (Futuyma and Moreno, 1988; Rohstein et al. 2002; Legros and Koella 2010). A high parasite specialisation increases host exploitation efficiency, however they are at the same time more susceptible to co-extinction since they rely strictly on one or few host species. On the other hand, generalist parasites have the advantage of lower extinction probability, but their decreased fitness in their hosts can reduce parasite establishment, adult survivorship and

¹ **Parasite specialisation:** Parasites adapt themselves to maximise their fitness in one or few closely related host species.

² **Generalist parasites:** They can parasitize a wide range of hosts.

fecundity in novel hosts (Gemmill et al. 2000; Poulin, 2005; Koprivnikar and Randhawa, 2013). A recent study, has estimated the probability of fish parasites to go co-extinct on the basis of its specificity and of the host extinction vulnerability and they found that specialized parasites use few, but less vulnerable hosts, reducing their co-extinction risk (Strona et al. 2013).

In many cases, the parasite counter-adaptations involve direct manipulation of host behaviour to increase probability of transmission and reproductive success of the parasite (Moore, 2013). If a parasite has a direct life cycle, survival and reproduction can be increased through social contact among conspecifics (Klein, 2005). For example, viruses that have direct life cycles typically cause an increased aggression and physical contact in the host population (Klein et al. 2004). Parasites relying on trophic transmission to complete their life cycles often induce modifications in their intermediate host's behaviour to increase their susceptibility to predation by final hosts (Lagrange et al. 2007; Poulin, 2013). For example, the acanthocephalan *Pomphorhynchus laevis* inhibits attraction to conspecifics in the amphipod intermediate host *Gammarus pulex* in order to increase predation by fish definitive hosts (Durieux et al. 2012) (Fig. 2).

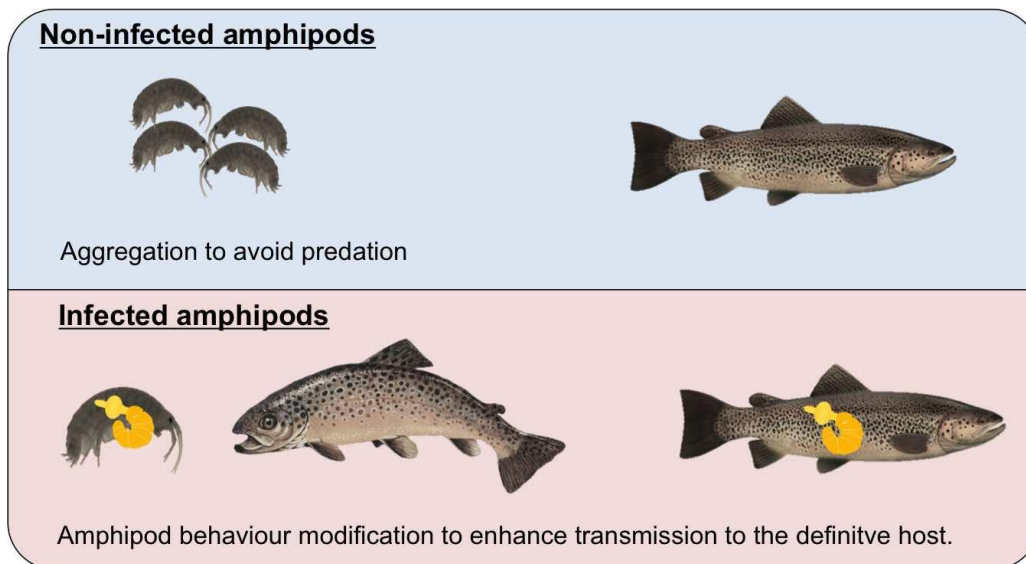


Figure 2. Behaviour modification of the amphipod *G. pulex* by the parasite *P. laevis* to facilitate transmission to the definitive host *Salmo trutta*.

1.1. Importance of parasitism in ecosystems

Parasites play an important role in population dynamics and community structure. Their impact reducing host fitness and therefore modifying competitive and trophic interactions among species have profound effects on the abundance of different species and the interactions between them (Hudson et al. 2006). Parasite species affect their hosts differently, thus they can mediate the co-existence of host species by harming some species more than others, which can contribute to the maintenance of host diversity (Poulin and Morand, 1999). For example, the amphipods *Corophium volutator* and *Corophium arenarium* coexist in nature and share a trematode parasite (*Microphallus claviformis*). *C. volutator* is the superior competitor, but it is also more sensitive to *M. claviformis*, presenting higher mortalities than *C. arenarium*. In this case, the parasite cancels the advantage of the superior competitor, which could lead to *C. arenarium* extinction and promotes the coexistence of the two congeners (Jensen et al. 1998).

Another way in which parasites can influence community structure is by indirectly modifying the functional importance of their host species in the community. Modifications of host behaviour or phenotype can lead to modifications of the host interactions with other species (Poulin and Morand, 1999). For example, rhizocephalan parasites modify the physiology of their crab host and infected crabs do not moult, which causes an increase of epibiotic invertebrates. Parasitized crabs become then functionally more important for the rest of the invertebrate community (Thomas et al. 1999).

Host modifications can also alter the host availability to other parasite species. Many parasite species interfere with the host immune system and immunosuppressed hosts can become more available to other parasite species (Wakelin, 1984).

Further evidence of parasite importance in shaping communities comes from biological invasion studies. Invader species tend to lose their co-evolved parasites in the process of invasion (enemy release hypothesis), increasing invaders performance over the native species (Torchin et al. 2003). Parasites that survive the host translocation can in turn infect naïve native host populations that have not developed defence mechanisms against them, which can eventually lead to local host extinction (Prenter et al. 2004; Hudson et al. 2006). For example, the native European crayfish *Austropotamobius pallipes* has been driven locally extinct as a result of transmission of the parasitic fungi *Aphanomyces astaci* from the

introduced North American crayfish *Pascifastacus leniusculus* (Holdich et al. 1991). Invader populations harbour significantly fewer parasites than native populations, since parasites from native hosts are often host specific and unable to spread to invaders (Dunn and Dick, 1998). Therefore, parasites play a major role mediating the interaction of invaders and native hosts and determining invaders success or failure (Prenter et al. 2004).

Finally, parasites are a valuable indicator of ecosystem health. Healthy ecosystems, that is, ecosystems that persist, maintain vigour, organisation and resilience to change, are reported to harbour a high diversity of parasites (Hudson et al. 2006). Given that pollution, climate change and other stressors may have impacts on populations of organisms, examination of parasite assemblages may reflect alterations in the food web structure and function, resulting from ecological disturbances to host distributions (Marcogliese, 2004, 2005). Some studies have reported that pollution and stress are often associated with a reduction in parasite richness due to the reduction of host species. However, some ectoparasite populations like protozoans or monogeneans may increase linked to a host's compromised immune response (McKenzie et al. 1995; McKenzie, 1999). Since parasites depend on host density for their transmission and some might depend on several host species, some studies have concluded that parasites are more sensitive to environmental degradation than their hosts (Marcogliese, 2005). Recently, Johnson et al. (2013) found that host and parasite richness interacted to negatively affect the transmission of the deadliest parasite *Ribeiroia ondatrae* in amphibians, emphasising the role of parasite communities regulating disease risk.

Despite increasing interest in host-parasite interactions, they remain poorly understood in reef fishes (Finley and Forrester, 2003). Understanding the relationship between environmental variables, parasitism and disease in reef fishes is highly important to understand the complexity and resilience of coral reef ecosystems (Sikkel et al. 2009). Lafferty et al. (2008b) showed that fish parasite richness was higher in a well-conserved atoll than in a heavily fished island, indicating the effect of some ecosystem disturbances on the parasite communities of coral reef fish. A recent study found a dramatically higher rate of infection of dermal parasites among coral reef fishes in Curaçao compared to other Caribbean sites like Belize and Mexico. Although responsible factors of this phenomenon remain unknown it is likely that the lack of predators in Curaçao contributes to a higher coral reef fish population and thus parasite abundance (Bernal et al. 2016). The study of host-

parasite interactions in cultured fish is also of crucial importance, since they are often more sensible to parasite outbreaks (Leung et al. 2013).

1.2. Parasitism in aquaculture

The emergence of infectious diseases in aquaculture is a major problem that causes severe financial losses through decreased production of farmed species or increased production costs, and important environmental consequences. For example, sea lice infections cause £15–30 million annual losses to Scottish salmon farms (Pike and Wadsworth, 2000).

Owing to the cost effective nature of aquaculture, fish may be cultured in densities more than 1000 times higher than under natural conditions. High fish densities increase the probability of contact between infected fish and susceptible hosts, facilitating the spread of pathogens (Pulkkinen et al. 2009). Besides, aquaculture is often linked to high stress levels in cultured animals and environment degradation, such as low water quality, low oxygen and high nitrogen levels, which reduce fish fitness and enhances infection by opportunistic pathogens (Pickering, 1998; Martin et al. 2010).

Another major problem of parasitic infections in aquaculture is the increase of virulence (Kennedy et al. 2016). Virulence is defined as the deleterious health effects of a pathogen infection in a host. Several studies have shown that aquaculture can create conditions that favour the development of highly virulent pathogens (Murray and Peeler, 2005; Pulkkinen et al. 2009). Parasites normally favour to exploit their hosts prudently to prolong infection and avoid killing the host, but they also need to exploit host resources to optimise reproduction and transmission. Therefore, there is a trade off between prudent host exploitation and rapid parasite reproduction (Figure 3). In aquaculture systems, the high number of hosts does not limit the fitness gain of increased infectiousness, and thus can lead to evolutionary increases in parasite virulence (Kennedy et al. 2016). Evolution of virulence theory predicts that virulence levels depend on host lifespans, so shortening the effective host lifespan, for example by compressing the rearing cycle duration, can also favour evolution of increased pathogen virulence (Nidelet et al. 2009). Selective breeding and inbreeding in broodstock populations are common in aquaculture leading to populations with limited genetic diversity. Host diversity can prevent parasite specialisation, however pathogen strains that specialise on low diversity populations can develop high virulence in those populations (Garamszegi, 2004;

Kennedy et al. 2016). Finally, continuous treatment against disease can also contribute to virulent form selection (Porco et al. 2005).

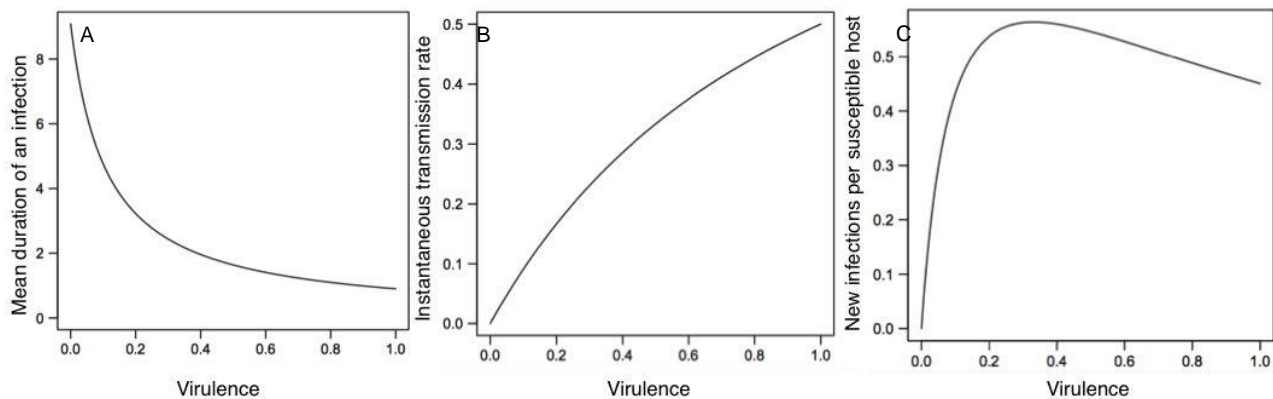


Figure 3. Illustration of a trade-off between parasite virulence and transmission adapted from Kennedy et al. (2015). Virulence induced host mortality shortens the duration of an infection (A), virulence increases the instantaneous transmission of infection (B). Result of the trade-off between virulence, transmission and infection duration can lead to a maximised pathogen fitness at intermediate levels of virulence in natural conditions (C).

Aquaculture pathogens can come from wild populations, however most of the agents of emerging diseases in aquaculture seem to come from other geographical areas or other host species via the artificial movement of live animals (Nylund and Jackobsen, 1995; Hedrick, 1996).

Warmer climate promotes higher pathogen proliferation and transmission rate, therefore mortality due to infectious diseases is likely to be higher at lower latitudes (Robar et al. 2010). The combination of overcrowding with warm temperatures provide the perfect conditions for disease outbreaks, thus aquaculture at low latitudes is exposed to more severe mortality and rapid progression of diseases (Mennerat et al. 2010; Leung et al. 2013). This is of particular significance, since lower latitudes are also the most vulnerable towards climate change. Understanding patterns of parasite distribution along geographical gradients might provide insights in the parasite responses to different climatic conditions and the risk of parasite outbreaks.

1.3. Distribution of parasite communities

Parasite community analysis often uses several metric indices such as prevalence, mean intensity, and richness that allow quantification of the infections. Prevalence is the proportion of infected hosts among all the hosts examined. Intensity is the mean number of parasites found within infected hosts. Parasite richness is the number of parasite species that are found in a given host species (Morand, 2015).

Animal parasite communities are highly dynamic and complex and they are generally influenced by host physiology, ecology and phylogeny (Muñoz et al 2006). Several studies have shown that larger hosts offer a larger number of habitats for parasite colonisation and thus, they may be expected to harbour richer parasite communities (Sasal et al. 1997; Sasal and Morand, 1998). Host diet seems to play an important role in parasite richness, especially in parasite with complex life cycle since hosts with broader diet ranges could acquire more trophically transmitted parasites³ than those with narrow, specialised diets (Simkova et al. 2001; Cirtwill et al. 2016). A recent study has shown that different foraging habits of animals can also expose them to encounter different ectoparasite species and thus be related to parasite richness and abundance (Luguterah and Lawer, 2015). Similarly, hosts with larger depth ranges or geographic ranges could have greater chances of encountering more parasite species, and therefore, present richer parasite faunas (Simkova et al. 2001; Luque et al. 2004; Garrido-Olivera et al. 2012). Although results from different studies do not fully agree, host phylogeny could play a role in parasite diversity and richness (Morand et al. 2000; Poulin et al. 2011; Alarcos and Timi, 2011). Inter-specific relationships within a host species (e.g. competition between parasite species) have been less studied, but are also potentially important in host-parasite relationships and structure of parasite communities (Hughes and Boomsma, 2004).

Rohde (1992) and Rohde et al. (1995) observed that marine fish from tropical latitudes typically harbour richer ectoparasite communities than fish from temperate latitudes, following the latitudinal diversity gradient. Although there is still some controversy, temperature seems a major force driving parasite richness and increasing their taxonomic complexity over evolutionary time, mediated by greater rates of speciation and host colonisation (Poulin, 2004; Garrido-Olivera et al. 2012). Although parasite biodiversity in

³ **Trophically transmitted parasites:** parasites transmitted through the food chain, by ingestion of parasitized preys.

coral reefs has not been evaluated, recent studies show high parasite diversity in coral reef fishes, suggesting an estimate of 10 parasite species per fish species (Justine, 2006; Justine et al. 2012)

Abundance of parasites may differ geographically coupled to environmental gradients following the "favourable centre" hypothesis, which states that a species reaches its highest abundance at the centre of its range, where optimal conditions for survival and reproduction exist. With increasing distance from this optimal site the conditions become less favourable and population size declines (Sagaring and Gaines, 2002). Similarity of parasite communities of the same host species tends to decrease exponentially with increasing distance between them, or increasing habitat differences (Poulin and Morand, 1999; Poulin et al. 2011). Distance decay in similarity results from three processes: species-sorting along environmental gradients, dispersal constraints imposed by topography, and intrinsic species differences in dispersal abilities (Soininen et al. 2007).

1.4. Chemical ecology in host-parasite interactions and host protection strategies from parasites

Chemical ecology is the study of chemicals involved in the interactions of living organisms and provides significant insights into the ecology and evolution of populations, from the organisation of communities and the function of ecosystems (Hay, 2009).

Parasites have evolved numerous strategies to evade host defences and succeed in infection, some of which involve chemical mediation. Social insects and their parasites are the best-studied group for chemical mediated host-parasite interactions. Social insects are known to produce semiochemicals (chemical substances involved in communication) in order to recognise their nest-mates and detect intruders. Many social parasites have evolved chemical mimicry in order to take advantage of their hosts without being noticed (Lenoir et al. 2001). For example, the larvae of the parasitic butterfly *Maculinea rebeli* produce mimetic hydrocarbons in the surface of their body that make the foraging *Mirmica schencki* workers recognize them as their own ant larvae and bring them to their nest brood chambers (Akino et al. 1999).

Chemical mediation in host-parasite interactions in the marine ecosystem is still poorly understood. However, some studies have found that parasites use chemical cues to identify their hosts. For example, the pathogenic bacteria *Vibrio coralliilyticus*, uses chemotaxis⁴ and chemokinesis⁵ to target the mucus of its stressed coral host, *Pocillopora damicornis* (Garren et al. 2014). Similarly, the pathogen *Flavobacterium columnare* is attracted by mucus from channel catfish (*Ictalurus punctatus*) (Klesius et al. 2008). Kallert et al. (2011) found that the nucleosides inosine and its 2'-deoxyinosine and guanosine from fish mucus attracted actinospores from 3 species of myxozoan and induced polar filament discharge and sporoplasm emission (myxozoan way of host invasion). Other studies have investigated the specificity of monogenean parasites and found that glycoproteins present in fish mucus induced the attachment of oncoramicidia (Yoshinaga et al. 2002; Ohashi et al. 2007).

Host defences against parasites usually involve the activation of the immune cascade involving the innate and adaptative immune systems. Innate immune initiation relies on the recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognizing receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors (Buchmann, 2001; Alvarez-Pellitero, 2008). Both TLR receptors and physical attachment of parasites (e.g. ectoparasites) initiate inflammatory responses, activating macrophages to produce cytokines, including tumour-necrosis factor (TNF), interleukins (IL-1, IL-6, IL-8), complement factor, lysozyme, antimicrobial proteins and peptides, the enzyme inducible nitric-oxide synthase (iNOS) and other oxidative factors (Jones, 2001; Alvarez-Pellitero, 2008). In ectoparasite infections, hypersecretion of mucus is the first response to infection. Excess of mucus interferes with parasite infection, making difficult parasite access to epithelial layers and removing parasites via mucus sloughing (Jones, 2001). Fish mucus contains several innate and humoral substances with biostatic or bioacidal activity such as immunoglobulin, complement, C-reactive proteins, lectins, lysozymes and antimicrobial peptides (Jones, 2001). Mucosal immune system also possesses lymphocytes with high specificity and memory capacities, which are able to differentiate commensals from pathogens (Gomez et al., 2013b).

The development of new techniques such as metabolomics and proteomics allows a more comprehensive study of chemical molecules from organisms, providing new opportunities to study differential expression of signalling molecules in organism's interactions (Kuhlisch and Pohnert, 2015; Brinchmann, 2016).

⁴ **Chemotaxis:** movement of an organism in response to a chemical stimulus.

⁵ **Chemokinesis:** Motile response of an organism to chemicals that induce a change in their migratory/swimming behaviour.

2. Metabolomics

Metabolomics is one of the most recent "omics" sciences, joining genomics, transcriptomics and proteomics as tools in global systems biology (Sumner et al. 2003). "omics" sciences use high-throughput technologies to study comprehensively biochemical and molecular processes within an organism, tissue or cell type (Weckwerth, 2003). Metabolomics is the comprehensive, qualitative, and quantitative study of the small molecules in an organism, the metabolome (Dunn et al. 2005). Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002). Combined with proteomics and genomics, metabolomics can help gain insight into systems biology by studying the metabolite alterations and their relationships to changes in gene expression, protein expression and enzyme activity (Want et al. 2010) (Figure 4). The metabolome of a sample provides a snapshot of the state of the organisms at that time under specific environmental conditions (Kosmidis et al. 2013). Metabolomics approaches prove useful to distinguish among individual signals and thus might serve in biomarker discovery in a wide range of topics, from disease biomarkers to indicators of short-term environmental changes in ecology (Wolfender et al. 2009).

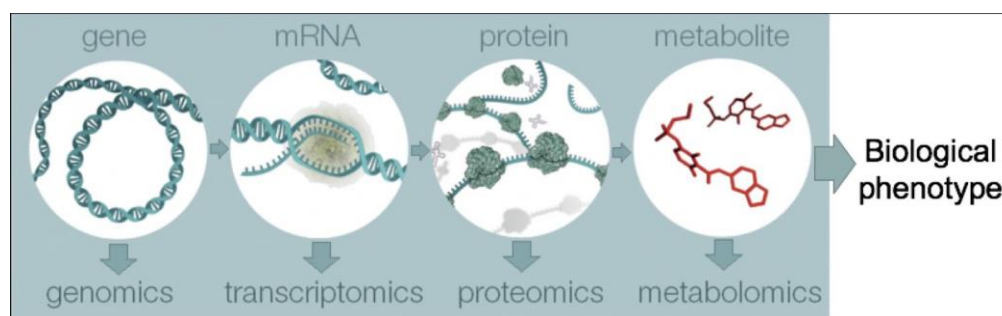


Figure 4. Overview from the 4 major "omics" sciences (adapted from www.ebi.ac.uk).

Metabolomics methodologies fall into two distinct groups, depending on the goal of the experiment. Targeted metabolomics is the measurement of defined groups of chemically characterised and biochemically annotated metabolites. Since targeted metabolomics analyses

known metabolites, sample preparation and analytical techniques can be optimised depending the chemical properties of these compounds. Untargeted metabolomics intends to study all the measurable analytes in a sample including chemical unknowns, therefore, special attention to sample preparation procedures and analytical techniques must be taken to avoid exclusion of metabolites (Fiehn, 2002; Roberts et al. 2012).

Most used analytical approaches in metabolomics include nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled techniques, including liquid chromatography (LC-MSⁿ), gas chromatography (GC-MS). NMR spectroscopy is the most common technique used to generate metabolomic data from biofluids and the main advantages is that it is a non-destructive, highly reproducible and fully quantitative analytic technique, taking only a few minutes per sample. MS methods, on the other hand, provide much higher sensitivity, enabling the quantitative measurement of a broader spectrum of metabolites at lower cost, however the injected part of the sample is destroyed during ionization (Reo, 2002; Kanani et al. 2008; Zhou et al. 2012; Kosmidis et al. 2013).

Metabolomic analysis produces large quantities of data that have to be pre-processed before being exploited by statistical analysis and chemometrics. Experiment design is of vital importance to have biologically significant data and that includes careful sampling design, extraction procedure, analytical analysis and data pre-processing and treatment. Due to the high number of samples analysed simultaneously, there is a high likeliness of bias introduction during sample analysis related to technical drift. Randomisation of samples or the use of a Latin square order in the analytical sequence can partly solve the problem, however, several algorithms have also been developed to correct technical drift in retention times between samples and deconvolute and align peaks, such as the free software XCMS. Once the data has been pre-processed (for example with XCMS), several univariate and multivariate statistics are often applied in order to extract the biological information of interest (Figure 5).

Metabolomics workflow

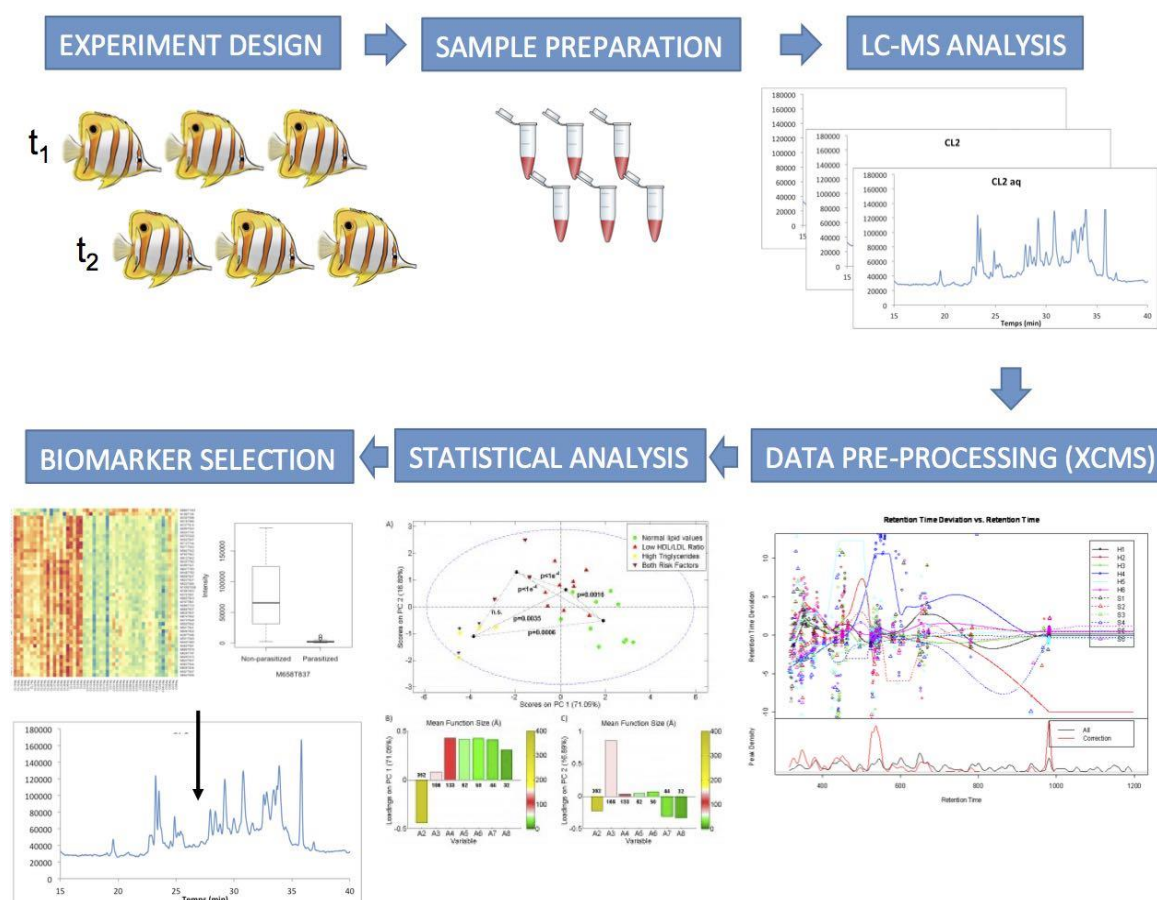


Figure 5. Metabolomics workflow for LC-MS.

2.1. Environmental metabolomics

Environmental metabolomics studies the organism's metabolic response to natural or anthropogenic stressors in its environment. Environmental metabolomics can be a powerful tool in 1) ecophysiology studies to unveil organism's biochemical responses to biotic or abiotic conditions such as effects of feeding behaviours on metabolism, 2) ecotoxicology to characterise metabolic responses of organisms to anthropogenic stressors, such as pollution and climate change and 3) to characterize the metabolites involved in organisms interactions (Viant, 2008).

One of the main advantages of metabolomics is the possible application to non-model species, since differences in metabolite fingerprints can be detected and analysed without knowing the metabolite identifications. However, *a posteriori* metabolite identification is an important step to biomarker discovery and identification of metabolic pathways that are altered in response to a biological perturbation, or discovering novel signalling compounds (Bundy et al. 2008).

In comparison to the large number of metabolomics studies in plants, human and model organisms (e.g. yeast or *Escherichia coli*), fewer metabolic profiles have been collected from aquatic organisms (Viant, 2007; Yanes et al. 2011; Quanbeck et al. 2012). Marine metabolomics, has however seen a rapid development in the last years, with increasing studies on wide-range of organisms including marine bacteria, micro and macroalgae, shellfish, sponges, corals and some fish species (Motti, 2012; Goulitquer et al. 2012).

Several studies have been performed to identify marine animal biochemical responses to stress caused by changing environmental conditions. For example, the metabolic response of mussels (*Mytilus edulis*) and marine copepods to acidification and increased temperature were studied by ¹H-NMR and LC-MS metabolomics (Ellis et al. 2014; Mayor et al. 2015). Metabolomics was also used to study metabolic response of Atlantic salmon (*Salmo salar*) and shrimp (*Litopenaeus vannamei*) to culture stressors (Karakach et al. 2009; Shock et al. 2013).

Marine metabolomics has also proven useful for chemotaxonomy purposes, to identify between different Homoscleromorpha sponges and *Symbiodinium* dinoflagellates species (Ivanisevic et al. 2010; Klueter et al. 2015). A recent study used metabolomics and proteomics to study chemical mediated competition in marine plankton (Poulson-Ellestad et al. 2014).

The use of metabolomic techniques to understand coral reef ecosystems has also exploded in the recent years. However most of the studies concentrate mainly on coral species or coral symbionts (He et al. 2014; Sogin et al. 2014; Klueter et al. 2015). He et al. (2014) studied the metabolic spatial variation of soft corals at two geographic locations in the South China Sea with NMR-bases metabolomic analysis. Sogin et al. (2014, 2016) showed that ¹H-NMR metabolomic profiles were species-specific among four species of reef-building corals and they studied the metabolic changes of *Pocillopora damicornis* under thermal and

acidification stress. A recent article (Quinn et al. 2016) used metabolomics to study reef benthic interactions between corals and algae and found that a bioactive lipid was involved in coral defence against algae. However, to date no study has yet assessed metabolic profiles and metabolic variability of coral reef fishes.

Objectives

Objectives

Fish parasites are an important part of ecosystems, however, in certain cases such as in aquaculture they cause severe disease outbreaks and host mortality. The objective of this Ph.D. is to gain knowledge of host-parasite interactions in coral reef fishes, through their study in both the natural and culture environments.

Parasite prevalence, intensity and virulence depend highly on parasite specificity. Therefore, the first objective of this thesis is to investigate parasite specificity and understand which factors shape it. To accomplish this goal we have analysed parasite specificity of monogenean parasites in the highly diversified Chaetodontidae family. We have surveyed the distribution of 13 species of dactylogyrids (Monogenea) among 34 butterflyfishes in the tropical Indo-Pacific islands (Chapter I), and we have studied the parasite biogeographical patterns in order to better understand evolution of host specificity among a large spatial scale (Chapter II).

Control of parasitic infections is crucial to host well-being. In aquaculture, spread of parasitic infections leads to high fish mortalities, and consequent important economic losses. Parasite control in aquaculture is mainly done by the use of synthetic persistent drugs, which have numerous side effects on fish and local biodiversity. The second and largest objective of the Ph.D. is to examine sustainable parasite control methods. This objective has been approached under two different directions. On one side, I have investigated parasite evasion methods in natural fish populations, which might provide new opportunities for parasite control in aquaculture. I observed that among the 34 butterflyfishes studied, only *Chaetodon lunulatus* was never parasitized by gill monogeneans. Therefore, I researched the species-specific factors in this species that might be related to this marked parasite absence. I have analysed the fish mucus, since it is the first surface of exchange between the fish and the environment and is known to be involved both in pathogen defence and parasite recognition (Chapter III). I then studied the gill mucus microbiome and metabolome of eight butterflyfish species (Chapter IV and V) and integrated the results in order to find specific microbial taxons and metabolites overexpressed in *C. lunulatus* (Chapter VI). On the other side, I assessed the use of medicinal plants as an alternative to chemotherapy in fish aquaculture (Chapter VII). I investigated the potential of several local Polynesian plants and algae against the two major pathogenic agents (*Vibrio harveyi* and *Tenacibaculum maritimum*), by

evaluating plant and algae antibacterial activities (Chapter VIII), studying the capacity of plants and algae to increase level of expression of two immune-related genes in orbicular batfish (*Platax orbicularis*) (Chapter IX) and finally testing *in vivo* efficacy of orally administered plants and algae in reducing infected (*Tenacibaculum maritimum*) orbicular batfish mortality (Chapter X).

HOST-PARASITE INTERACTIONS IN CORAL REEF FISH

- General introduction
- Objectives

PART I:

Fish-parasite interactions in the wild: Butterflyfish – monogenean interactions

Scientific context and model species

CHAPTER I: Gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfishes from tropical Indo-west Pacific Islands.

CHAPTER II: Biogeography of coral reef fish parasites: monogeneans from butterflyfishes in the West Pacific Tropical Islands.

CHAPTER III: External (skin and gills) fish mucus; biological and ecological importances, what is known and what is next?

CHAPTER IV: Characterisation of the gill mucus microbiome of four species of butterflyfish: a bacterial diversity reservoir in coral reefs.

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CHAPTER VI: Do mucus microbiome and metabolome interact to prevent ectoparasitic infection in a coral reef fish?

PART II:

Fish-parasite interactions in aquaculture: Orbicular batfish – bacterial interactions

Scientific context and model species

CHAPTER VII: Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives.

CHAPTER VIII: Antibacterial activity of local Polynesian plants and algae against fish pathogenic bacteria.

CHAPTER IX: Effects of local Polynesian plants and algae on growth and expression of two immune-related genes in orbicular batfish (*Platax orbicularis*).

CHAPTER X: Effects of local Polynesian plants and algae against *Tenacibaculum maritimum* infections in orbicular batfish (*Platax orbicularis*).

- Conclusion and perspectives
- Bibliography

Part 1



**Fish-parasite interactions in the wild :
Butterflyfish-monogenean interactions**

Scientific context and model species

Scientific context and model species

Butterflyfishes (Order: Perciformes, family: Chaetodontidae) are a diverse and conspicuous coral reef fish family widely distributed in all tropical seas. They are almost exclusively associated with coral reefs, but a few species are found in estuarine areas and over soft bottom habitats (Roberts and Ormond, 1992). There are 130 species of butterflyfish described to date, belonging to 10 genera (Tricar and Boyle, 2015). Although, most butterflyfish species are easily distinguished due to their colorful patterns, several hybrids (34% of the species) are known to occur (Montanari et al. 2014). Butterflyfish possess a highly compressed body, a small protractile mouth with brush-like teeth and their maximum lengths vary from 10 to 30 cm depending on the species. Most are highly social species, which form long-term monogamous pairs with territorial and "home-ranging" behaviors. The few planktivorous butterflyfish species tend to form larger associations or schools (Roberts and Ormond, 1992; Pratchett et al. 2013). Recent studies have showed that a high number of butterflyfish species produce sounds during social interactions (Tricar and Boyle, 2015).



Figure 1. Photographs of two butterflyfish species. (A) *Forcipiger longirostris* (B) *Chaetodon lunulatus*

Phylogenetic studies of the family Chaetodontidae show a robust phylogeny consisting of the bannerfish clade, which includes genus like *Forcipiger*, *Heniochus* and *Hemitaurichthys*; and the butterflyfishes, which includes the genus *Prognathodes* and *Chaetodon*. The genus *Chaetodon* itself is distributed within 4 clades (Littlewood et al. 2004; Fessler and Westneat, 2007) (Figure 2).

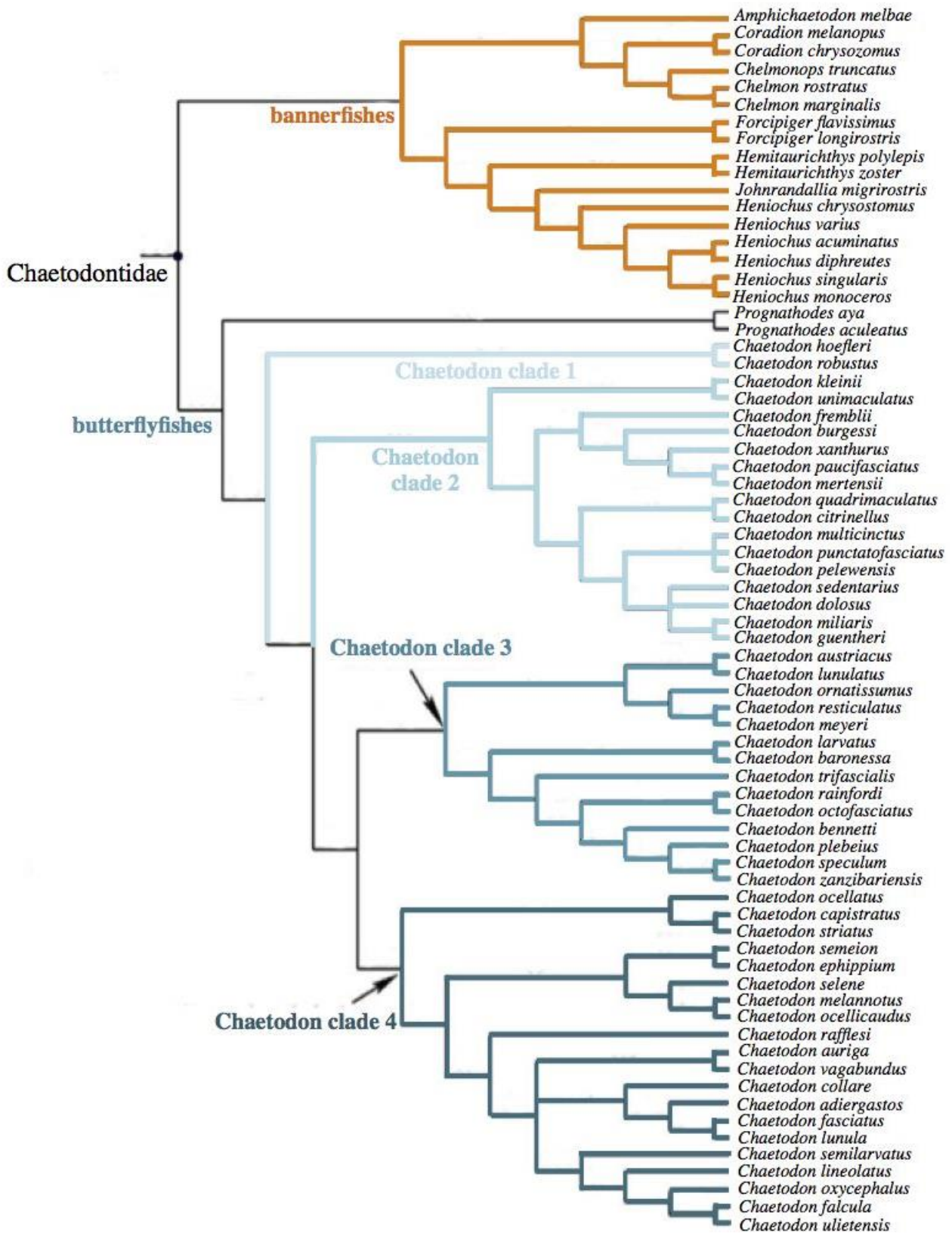


Figure 2. Phylogenetic tree of the Chaetodontidae family (adapted from Fessler and Westneat, 2007).

Butterflyfish consume a wide variety of prey including scleractinian corals, alcyonacean corals, algae, polychaetes, crustaceans and plankton. However, most species feed primarily, if not exclusively, on scleractinian corals. Butterflyfishes can be categorized upon their diet as obligate corallivores, facultative corallivores, omnivorous and planktivorous (Pratchett 2005, Pratchett et al. 2014a).

Different butterflyfishes also exhibit different levels of diet specialization. For example, *Chaetodon trifascialis* is among the most specialized of all coral reef fish, feeding almost exclusively on one single coral species, *Acropora hyacinthus*. On the other side, *Chaetodon lunulatus* is among the most generalist obligate corallivore (Pratchett, 2005). A recent study showed that specificity of a corallivorous butterflyfish (*Chaetodon triangulum*) varied depending level of coral coverage, with a lower feeding specificity and a higher territory size in low coral cover sites (Chandler et al. 2016). Several butterflyfish species (e.g. corallivorous species or with diets depending on coral-rich habitats) are sensible to coral loss and readily respond to changes in reef environments, with marked declines of some butterflyfish species upon extensive coral depletion (Pratchett et al. 2006b, 2011, 2014b).

Although butterflyfish are one of the best-studied coral reef fish, study of their parasite communities remains limited. Parasites, are an intrinsic part of organisms, and therefore play a major role in the evolution, adaptation and success or extinction of host species. A better comprehension on butterflyfish parasite communities is therefore important to better understand their stressors and possible adaptations. Also, since butterflyfish ecology and phylogeny has been extensively studied, butterflyfishes provide a good model to study parasite communities in large geographic areas.

Butterflyfishes are parasitized by several endo- and ectoparasite species. Morand et al. (2000) analyzed butterflyfishes endoparasite richness and identified 10 species of digeneans, 2 nematode species and 1 Acanthocephalean species. Some ectoparasites that have been found in butterflyfish include isopods (family Gnathiidae and Cymothoidae) (Meadows and Meadows, 2003; Coile and Sikel, 2013), transversotrematid trematodes (Cribb et al. 2014), faustulids (Diaz et al 2013, 2015), and monogeneans (Plaisance and Kritsky 2004, Plaisance et al. 2004).

Monogenean parasites (Plathelminthes, Bychowsky 1937) are direct cycle ectoparasites mainly found on fish skin or gills. Monogeneans are a morphologically and ecologically

diverse group, with several thousand species described (Poulin, 2002). They can be divided into two major groups based on the complexity of their attachment structures (haptor): the monopisthocotyleans, which have hook-like organs on their haptors, and the polyopisthocotyleans, which use clamp-like structures for attachment (Reed et al. 2009, Figure 3). Most monogeneans are oviparous, releasing heavy ciliated free-swimming larvae (oncoramicidium) that are able to seek their hosts actively over short distances. Monogeneans tend to be host specific, one monogenean species infecting only one or few host species (Sasal et al. 1999). Buchmann and Linderstrøm (2002) proposed that monogenean specificity must be governed by factors on the host surface. It has been suggested that chemical stimuli emitted from the host could attract the parasites and initiate certain behavioural and physiological changes in the parasite. Hirazawa et al. (2003) found that *Heterobothrium okamotoi* oncomiracidia attached preferentially to skin mucus of its host species (tiger puffer, *Takifugu rubripes*) when several fish skin mucus were offered, and proposed that pH of the host mucus could be used by the parasite to identify the host. Two other studies found that capsalid oncomiracidia attachment to the hosts was induced by skin and mucus extracts, and they identified a host glycoprotein as the possible responsible of the recognition (Yoshinaga et al. 2002; Ohashi et al. 2007).

Butterflyfishes are parasitized by gill monogeneans belonging to the family Dactylogyridae (Yamaguti, 1963). A total of 15 dactylogyrid species belonging to the genus *Haliotrema* and *Euryhaliotrematoides* have been described to date in the Chaetodontidae family (Plaisance and Kritsky, 2004; Plaisance et al. 2004a). *Haliotrema* is a highly diversified genus that contains more than 100 species and is found in 6 orders of teleost fish. Six *Haliotrema* species are known to parasitize butterflyfishes. *Euryhaliotrematoides* is only found on fish from the Chaetodontidae family (Figure 3). To date, 9 species have been described in the genus *Chaetodon*, *Forcipiger* and *Heniochus* (Plaisance and Kritsky, 2004; Plaisance et al. 2004, 2005).

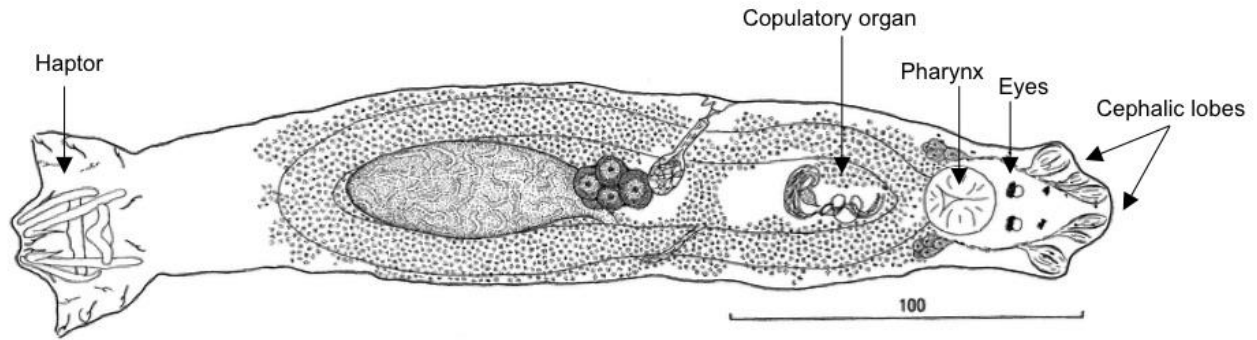
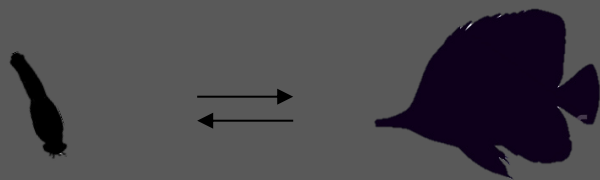


Figure 3. Drawing of *Euryhaliotrematoides grandis*, monopisthocotylean monogenean (adapted from Plaisance and Kritsky, 2004).

In this first part of the thesis I have studied parasite specificity of butterflyfish monogenean parasites and I have investigated which factors shape it. I have studied the monogenean communities of 34 butterflyfish species from the Indo West-Pacific and I have identified 13 dactylogyrid species. I have studied the dactylogyrid intensity, prevalence, richness and diversity in each butterflyfish species as well as the taxonomic composition of their dactylogyrid assemblages. I then investigated the host factors that could be involved in the observed patterns (phylogenetic signal, size, diet, geographic range and depth range). I have investigated the biogeography of dactylogyrid parasites in the West-Indo Pacific and I have assessed the effect of locations geographic distance, similarity in host communities and environmental similarities on the differences of parasite communities. *Chaetodon lunulatus* was the only butterflyfish species never found parasitized by gill monogeneans. Therefore, I studied which are the species-specific factors in this species that might be related to this marked parasite absence. I have studied the fish mucus, since it is the first surface of exchange between the fish and the environment and is known to be involved both in pathogen defence and parasite recognition. I then studied the gill mucus microbiome and metabolome of several butterflyfish species and integrated the results in order to find specific microbial taxons and metabolites overexpressed in *C. lunulatus*.



CHAPTER I

Gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfish from tropical Indo-west Pacific Islands

Reverter, M., Cutmore, S.C., Bray, R., Cribb, T.H., Sasal, P. Gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfishes from tropical Indo-West Pacific Islands. *Parasitology. In press.* (IF = 3.0)

Gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfishes from tropical Indo-west Pacific Islands.

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Summary

We studied the monogenean communities of 34 species of butterflyfish from the tropical Indo-west Pacific, identifying 13 dactylogyrid species (including 2 species that are presently undescribed). Monogenean assemblages differed significantly between host species in terms of taxonomic structure, intensity and prevalence. Parasite richness ranged from 0 (*Chaetodon lunulatus*) to 11 (*C. auriga*, *C. citrinellus* and *C. lunula*). Host specificity varied between the dactylogyrids species, being found on 2-29 of the 34 chaetodontid species examined. Sympatric butterflyfish species were typically parasitised by different combinations of dactylogyrid species, suggesting the existence of complex host-parasite interactions. We identified six clusters of butterflyfish species based on the similarities of their dactylogyrid communities. Dactylogyrid richness and diversity were not related to host size, diet specialisation, depth range or phylogeny of butterflyfish species. However, there was a weak positive correlation between monogenean richness and diversity and host geographical range. Most communities of dactylogyrids were dominated by *Haliotrema aurigae* and *H. angelopterum*, indicating the importance of the genus *Haliotrema* in shaping monogenean communities of butterflyfishes. This study casts light on the structure of the monogenean communities of butterflyfishes, suggesting that the diversity and complexity of community structures arises from a combination of host species-specific parameters.

Key words: monogenean, dactylogyridae, butterflyfish, parasite community, specificity, host-parasite interactions

Key findings

- Dactylogyrid communities of 34 species of butterflyfish were studied.
- Intensity and prevalence of dactylogyrids varied between sympatric butterflyfishes.
- *C. lunulatus* was the only butterflyfish species that was never found parasitised.
- Sympatric butterflyfishes were parasitised by different combinations of dactylogyrid species.
- Results suggest that the complexity of dactylogyrid communities arise from host-species specificities.

1. Introduction

Parasites are an essential part of every ecosystem, acting as agents of natural selection and contributing to community and ecosystem organisation. Parasites modify host behaviour and generate pressure for selection and adaptation by their hosts (Hatcher et al. 2012; Gómez and Nichols, 2013). Given that parasites rely on their hosts for resources, changes in the host population also affect parasite communities, these interactions leading to on-going host-parasite coevolution (Quigley et al. 2012).

Parasite assemblages are highly complex and dynamic, resulting from the combination of parasite physiological traits and ecology, and multiple host ecological parameters and phylogenetic histories (Muñoz et al 2006). For fishes, several studies have shown that parasite communities are influenced by host size, diet, geographic range and phylogenetic affiliation (Sasal et al. 1997; Morand et al. 2000; Simkova et al. 2001). Inter-specific relationships within a host species (e.g. competition between parasite species) have been less well studied, but are also potentially important in host-parasite relationships and structure of parasite communities (Hughes and Boomsma, 2004). Parasite specificity can vary dramatically between species, and specificity patterns and drivers are still being explored (Simkova et al. 2006). However, factors associated with recognition and selection of the host certainly play a major role in the structure of parasite assemblages (Buchmann and Linderstrøm, 2002). Therefore, studies of parasite communities should also take into account the possibility of chemical cues or other factors playing major roles in parasite specificity.

Butterflyfishes (family Chaetodontidae) are a diverse and emblematic family of coral reef fishes distributed widely in all tropical seas. It has been proposed that butterflyfish might be used to monitor health status of coral reefs or as a proxy of total reef fish assemblage (Kulbicki and Bozec, 2005). Butterflyfish ecology and behaviour has been extensively studied, and they can consume a variety of prey, including algae, polychaetes, crustaceans and coral (Pratchett, 2005). Most species of butterflyfish are associated with coral cover, and some studies have shown butterflyfish vulnerability to coral loss (Pratchett et al. 2011). Although some studies have investigated parasite communities on butterflyfishes in the tropical Indo-west Pacific (TIWP), little is known about the ectoparasite assemblages among different species of butterflyfish (Morand et al. 2000; Yong et al. 2013; Cribb et al. 2014; McNamara et al. 2014). Because of butterflyfishes high diversity and abundance in coral reefs, they provide a good model to study the arrangement of parasite communities between phylogenetically and ecologically similar host species along large geographical scales. Also, the knowledge on butterflyfishes phylogeny and ecology can help in the study of the underlying factors shaping parasites communities.

Platyhelminth monogeneans have direct life cycles, living predominantly on the gills and skin of fishes (Kearn, 1994). Butterflyfishes are parasitised by gill monogeneans belonging to the family Dactylogyridae, with a total of 15 species reported from chaetodontids to date (Plaisance and Kritsky, 2004; Plaisance et al. 2004). Dactylogyrids are the primary monogenean group found on this host family; a few capsalids are the only other known monogeneans known to infect chaetodontids (Bullard et al. 2000). Although some recent studies have revised the classification of the monogenean fauna of butterflyfish and their molecular phylogeny (Plaisance and Kritsky, 2004; Plaisance et al. 2004; Plaisance et al. 2005; Kritsky, 2012), little is known about monogenean community structure.

In this study we aim to better understand the structure of dactylogyrid communities (intensity, prevalence, diversity and taxonomic composition) infecting multiple butterflyfish species in the TIWP and the factors shaping them. Since dactylogyrids are direct cycle parasites, we could expect that sympatric butterflyfishes with close ecologies and phylogeny would present similar parasitism levels and taxonomic compositions (Tavares and Luque, 2008), unless strong species-specific factors between different butterflyfishes play a major role in structuring dactylogyrid communities. Firstly, we studied variation of dactylogyrid intensities, prevalence, richness and diversity among 34 butterflyfish species and we analysed

the influence of host factors (phylogenetic signal, size, diet, geographic range and depth range) on the parasitism differences between host species. Secondly, we studied the taxonomic composition of dactylogyrid communities among the 34 butterflyfishes, and we identified different groups of butterflyfish species with similar dactylogyrid assemblages. We studied the relative effects of host phylogenetic, ecological (body size and feeding behavior) and geographic distribution (geographic range and depth range) similarities on the taxonomic structuring of dactylogyrid assemblages between butterflyfish species using multivariate regression on distance matrices. Correlations between parasites species were used to see if there were strong antagonisms or synergisms between dactylogyrid species that could influence different taxonomic composition between hosts.

2. Materials and methods

2.1. Sampling

Thirty-four species of butterflyfish ($n = 560$) were collected at 8 localities in the Pacific: Palau, Wallis and Futuna, Lizard Island (Australia), and the 5 archipelagos in French Polynesia (Society Islands, Tuamotu Islands, Austral Islands, Gambiers Islands and Marquesas Islands). Butterflyfish were also sampled from a single locality in the Indian Ocean, from Ningaloo Reef on the west Australian coast (Fig. 1).

Adult butterflyfish were captured, put in individual plastic bags with seawater and brought immediately to a laboratory for dissection. Fish total and standard lengths were measured and gills were removed and fixed in 70% ethanol. Dactylogyrids were removed from the gills using a stereo microscope and were mounted on glass slides in Malmberg's ammonium picrate-glycerine.

All dactylogyrids from each individual fish were observed under a compound microscope, and parasite species and number were recorded. Dactylogyrid species determination was according to published literature (Plaisance and Kritsky 2004, Plaisance et al. 2004; Kritsky, 2012) and was based on the morphological characters of the sclerotised structures (the haptor and the reproductive organs).

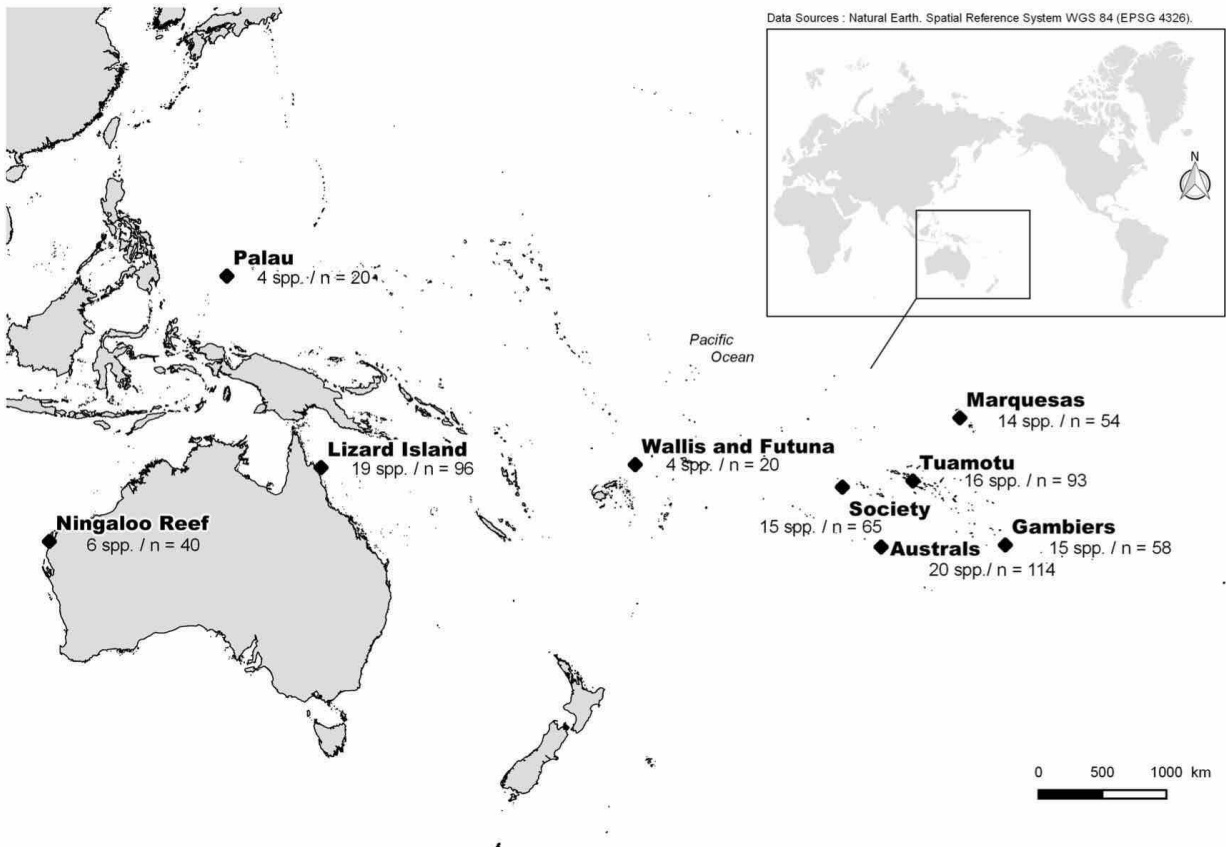


Figure 1. Map of sampling sites in the tropical Indo-west Pacific, with the number of species and total fish sampled per site.

2.2. Data analysis

2.2.1. Dactylogyrid intensity, prevalence, richness and diversity

Counts of total monogeneans per fish were used to calculate intensity and prevalence for each host species. Normality of parasite intensities and prevalence (Saphiro-Wilk test) and homogeneity of variances (Levene tests) were tested and not satisfied, thus non-parametric tests were used in this data analysis sub-section. Non-parametric Kruskal-Wallis test and Kruskal post-hoc test were performed in order to detect parasite intensity differences between chaetodontid species. Host species intensity and prevalence were displayed using boxplots, where the median, first and third quartile were plotted.

Detailed counts of each dactylogyrid species per fish were used to calculate parasite richness, evenness and diversity [Shannon (H)] for each species of butterflyfish. Species

accumulation curves for each butterflyfish species were generated with the “vegan” package for R, and expected richness were calculated using the “exact” method.

The Chaetodontidae phylogenetic tree was reconstructed under the newick format using the phylogenetic tree from Fessler and Westneat (2007), and the package “phytools” for R was used to read the tree. Since phylogenetic tree branch lengths were not available, phylogenetic signal on the parasite intensity and richness was measured using the Abouheif-Moran test, which has been recently defined as a powerful alternative to the K statistic when branch lengths are not accurate (package “adephylo” for R) (Abouheif, 1999; Pavoine et al. 2008; Pavoine and Ricotta, 2013).

Linear regression and Spearman correlation analyses were performed to analyse the influence of host factors (size, host geographical range, depth range and feeding behaviour) on parasite intensity and parasite richness. Host geographical range (km²), depth range and feeding behaviour data were obtained from FishBase (Froese and Pauly, 2016) and the IUCN Red List (IUCN, 2016). Feeding behaviour was categorized into the following groups depending on the level of specificity: 1 = specialist (feeding on restricted species), 2 = corallivore (feeding mostly on scleractinians), 3 = omnivore (feeding on a wide variety of organisms including corals, algae and invertebrates).

Taxonomic composition of dactylogyrid assemblages

Analyses of the dactylogyrid taxonomic composition were done using detailed counts of each dactylogyrid species per fish. A hierarchical cluster analysis («hclust» package for R, Euclidian distance) was used to identify groups of butterflyfish species with similar taxonomic composition of dactylogyrids. The "Average" algorithm was chosen after analysis of the cophenetic correlation coefficients (Pearson correlation between the cophenetic distances calculated on cluster branches and the parasite dissimilarity matrix) (Kulbicki et al. 2013). The Kelley-Gardner-Sutcliffe (KGS) penalty function was used to prune the dendrogram (Bottegoni et al. 2006). A Principal Component Analysis (PCA) was employed to analyse the correlations between the dactylogyrid species with the use of a correlation circle.

Multivariate regression on distance matrices (function MRM, package “ecodist” for R) was used to evaluate the relative effects of host phylogeny, host ecology and host geographical distribution on the taxonomic composition of dactylogyrid assemblages among

different butterflyfish species. A matrix of phylogenetic distances between pairs of Chaetodontidae species was obtained using the `distTips` function (“`adephylo`” R package, `method = nNodes`) from the reconstructed phylogenetic tree (see the ‘Dactylogyrid intensity, prevalence, richness and diversity’ section). The chaetodontid ecology (host maximum size and feeding behavior) and geographical distribution (geographic range and depth range) matrices of dissimilarity were obtained using the `vegdist` function (“`vegan`” R package, Euclidean distance).

3. Results

3.1. Species identification

Thirteen dactylogyrid species were identified based on the morphology of the sclerotised structures, two of which are undescribed (*Euryhaliotrema grandis* (Mizelle & Kritsky, 1969), *Euryhaliotrema annulocirrus* (Yamaguti, 1968), *Euryhaliotrema triangulovagina* (Yamaguti, 1968), *Euryhaliotrema pirulum* (Plaisance & Kritsky, 2004), *Euryhaliotrema microphallus* (Yamaguti, 1968), *Euryhaliotrema berenguelae* (Plaisance & Kritsky, 2004), *Euryhaliotrema aspistis* (Plaisance & Kritsky, 2004), *Euryhaliotrema cribbi* (Plaisance & Kritsky, 2004), *Haliotrema angeloferum* Plaisance, Bouamer & Morand, 2004, *Haliotrema aurigae* (Yamaguti, 1968), *Haliotrema scyphovagina* (Yamaguti, 1968), *H. sp. A* and *H. sp. B*).

H. sp. A is most similar to *H. aurigae*, having similar haptors, but differed in the possession of a much smaller, well sclerotised copulatory organ. *H. sp. B* is most similar to *H. angeloferum*, with both morphotypes possessing fenestrated dorsal anchors, but the undescribed species had considerably different copulatory organs. These species will be described elsewhere. Three described dactylogyrid species were not found in the butterflyfish sampled: *Haliotrema monoporosum* Pan and Zhang, 2000, described from *Chaetodon wiedeli* Kaup, 1863 in the South China sea; *Haliotrema brevicirrus* Zhukov, 1980, from *C. striatus* Linnaeus, 1758, *C. capistratus* Linnaeus, 1758, and *C. ocellatus* Bloch, 1787, from the Gulf of Mexico and *Haliotrema spirale* Yamaguti 1968 from *C. fremblii* Bennett, 1828, from Hawaii.

3.2. Dactylogyrid intensity, prevalence, richness and diversity

Monogenean prevalence and intensity was found to be highly variable between sympatric butterflyfish species. Fifteen chaetodontid species were found to always be parasitised by dactylogyrids, whereas *Chaetodon lunulatus* (n = 30) was the only species that was never found to be parasitised (Figure 2). Kruskal-Wallis test and Kruskal post-hoc test allowed the identification of a group of 10 butterflyfish species (*C. lunulatus* Quoy and Gaimard, 1825, *C. ephippium* Cuvier, 1831, *C. baronessa* Cuvier, 1829, *C. pelewensis* Kner, 1868, *Hemitaurichthys polylepis* Bleeker, 1857, *C. semeion* Bleeker, 1855, *C. speculum* Cuvier, 1831, *C. ulietensis* Cuvier, 1831, *Heniochus chrysostomus* Cuvier, 1831 and *C. unimaculatus* Bloch, 1787) with low dactylogyrid intensities that differed significantly ($P < 0.05$) from eight fish species that had high intensities and were always parasitised (*C. vagabundus* Linnaeus, 1758, *Chelmon marginalis* Richardson, 1846, *C. ornatissimus* Cuvier, 1831, *C. citrinellus* Cuvier, 1831, *C. reticulatus* Cuvier, 1831, *C. quadrimaculatus* Gray, 1831, *C. declivis* Randall, 1975, and *H. acuminatus* Linnaeus, 1758) (Figure 3).

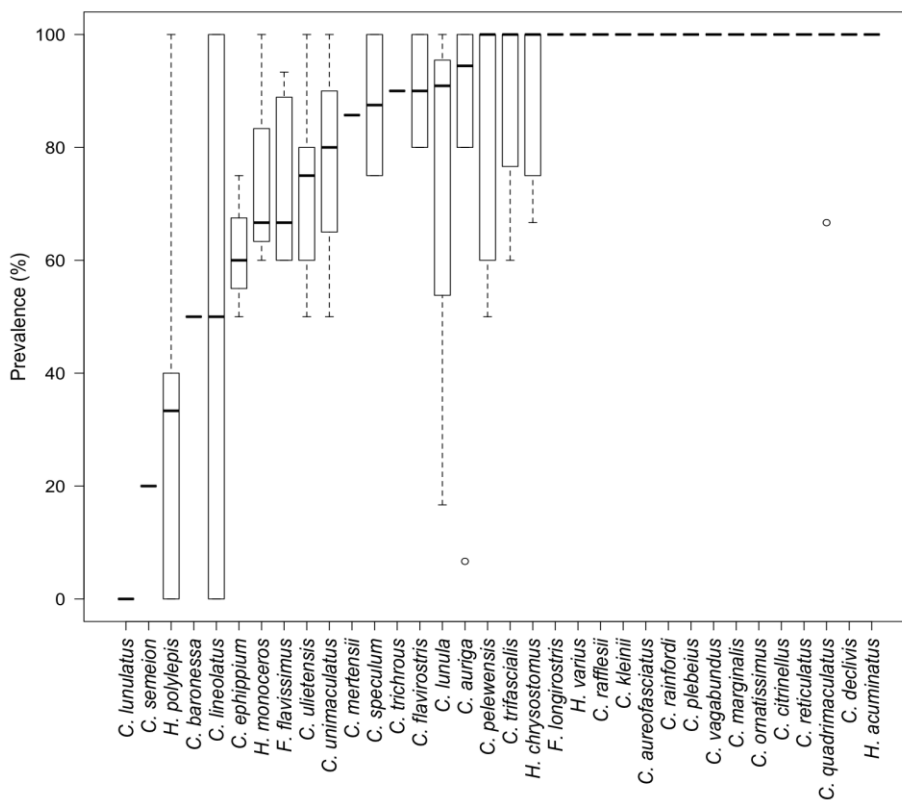


Figure 2. Dactylogyrid prevalence for the butterflyfish species analyzed. Median, first and third quartile are plotted. White dots represent outlier samples.

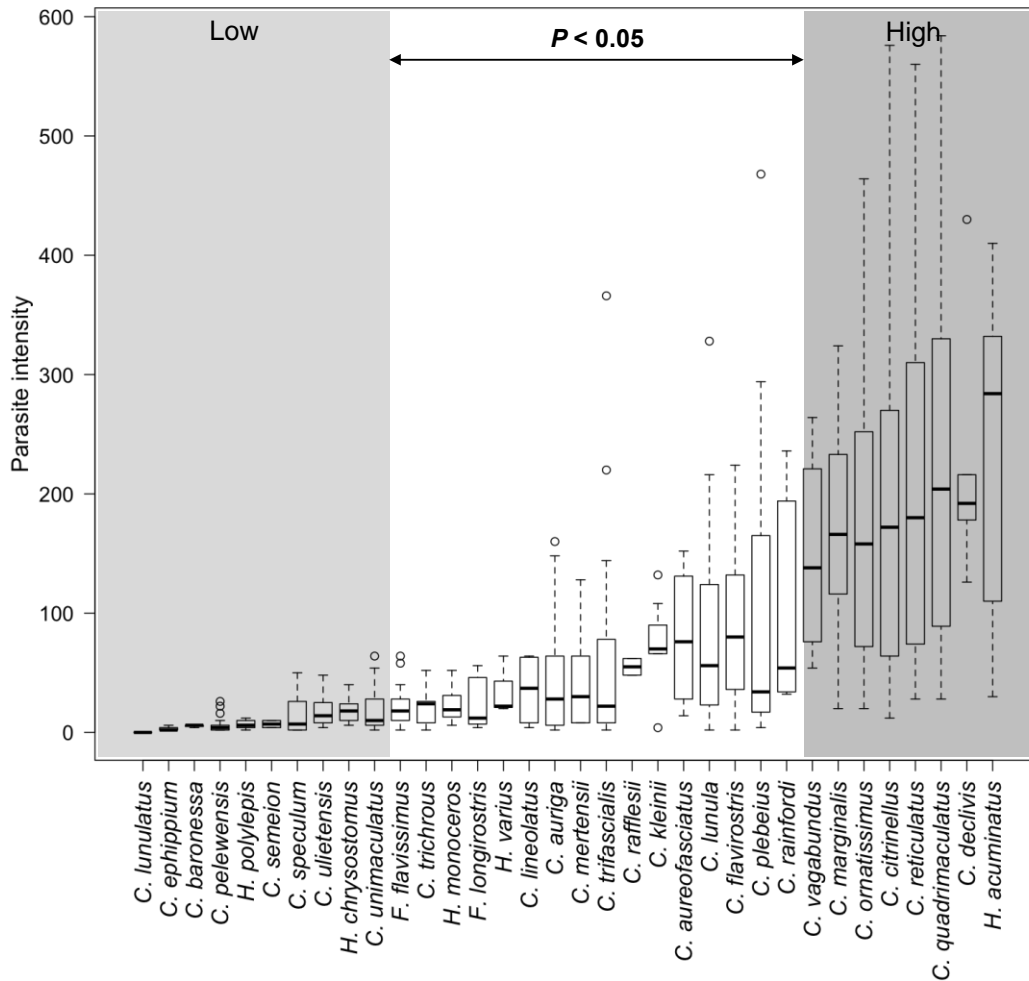


Figure 3. Dactylogyrid intensities for the butterflyfish species analyzed. Median, first and third quartile are plotted. White dots represent outlier samples. The grey areas have been determined as statistically different by kruskal post-hoc test.

Species accumulation curves for dactylogyrid species for each chaetodontid species showed that despite differences in sampling effort between fish species, parasite richness seems well described for nearly all fish species (Supp. material). The highest monogenean richness for any chaetodontid analysed in this study (11 spp.) was found for *C. citrinellus*, *C. auriga* Forsskål, 1775 and *C. lunula* Lacépède, 1802, followed by *C. trifascialis* Quoy and Gaimard, 1825, and *C. ulietensis*, each with 10 species. The lowest richness (other than the complete absence of infection for *C. lunulatus*), was observed on *C. semeion*, which was parasitised by a single monogenean species. The highest Shannon's diversity (H) was found on *F. flavissimus* Jordan and McGregor, 1898 ($H = 1.82$), followed closely by *C. trifascialis*

($H = 1.81$) and *C. pelewensis* (1.76). The lowest dactylogyrid Shannon's diversity (besides *C. semeion*, $H = 0$) was found on *C. quadrimaculatus* ($H = 0.17$). Evenness (E) followed the same trend as diversity, with *F. flavissimus* having the highest evenness ($E = 0.87$) and *C. quadrimaculatus* the lowest ($E = 0.10$) (Table 1).

Fish species	Parasite richness	Evenness (E)	Diversity index (H)
<i>Chaetodon aureofasciatus</i>	5	0.68	1.33
<i>Chaetodon auriga</i>	10	0.57	1.38
<i>Chaetodon baronessa</i>	2	0.59	0.41
<i>Chaetodon citrinellus</i>	11	0.67	1.6
<i>Chaetodon declivis</i>	5	0.68	1.1
<i>Chaetodon ephippium</i>	4	0.76	1.05
<i>Chaetodon flavirostris</i>	9	0.68	1.49
<i>Chaetodon kleinii</i>	7	0.68	1.33
<i>Chaetodon lineolatus</i>	5	0.59	0.95
<i>Chaetodon lunula</i>	10	0.48	1.15
<i>Chelmon marginalis</i>	5	0.45	0.72
<i>Chaetodon mertensii</i>	6	0.28	0.5
<i>Chaetodon ornatissimus</i>	8	0.46	0.95
<i>Chaetodon pelewensis</i>	8	0.85	1.76
<i>Chaetodon plebeius</i>	9	0.48	1.06
<i>Chaetodon quadrimaculatus</i>	6	0.1	0.17
<i>Chaetodon rafflesii</i>	7	0.74	1.44
<i>Chaetodon rainfordi</i>	8	0.76	1.59
<i>Chaetodon reticulatus</i>	8	0.39	0.81
<i>Chaetodon semeion</i>	1	NA	0
<i>Chaetodon speculum</i>	3	0.2	0.22
<i>Chaetodon trichrous</i>	3	0.7	0.77
<i>Chaetodon trifascialis</i>	10	0.79	1.81
<i>Chaetodon ulietensis</i>	10	0.68	1.56
<i>Chaetodon unimaculatus</i>	7	0.5	0.97
<i>Chaetodon vagabundus</i>	7	0.78	1.52
<i>Forcipiger flavissimus</i>	8	0.87	1.82
<i>Forcipiger longirostris</i>	7	0.73	1.42
<i>Heniochus acuminatus</i>	3	0.53	0.58
<i>Heniochus chrysostomus</i>	8	0.64	1.34
<i>Heniochus monoceros</i>	7	0.66	1.28
<i>Heniochus varius</i>	3	0.18	0.51
<i>Hemitaurichthys polylepis</i>	3	0.46	0.2

Table 1. Parasite richness, diversity (H) and evenness (E) of the 33 butterflyfish species parasitized by dactylogyrids.

Host phylogenetic signal and ecological factors (size, geographical range, depth range and feeding behaviour) were studied in order to estimate their influence on the differences of parasitism intensities and richness between different butterflyfish species. Host phylogenetic signal (Abouheif-Moran test) was non significant for neither parasite intensity ($P = 0.468$) nor

richness ($P = 0.276$). Size, depth range, feeding behaviour (specificity level) did not correlate with either parasite intensity or parasite richness among different chaetodontid species. Host geographical range displayed a moderate positive Spearman correlation ($\rho = 0.46$; $P = 0.01$) with parasite richness. Linear regression showed also a positive correlation ($r^2 = 0.16$) (Figure 4).

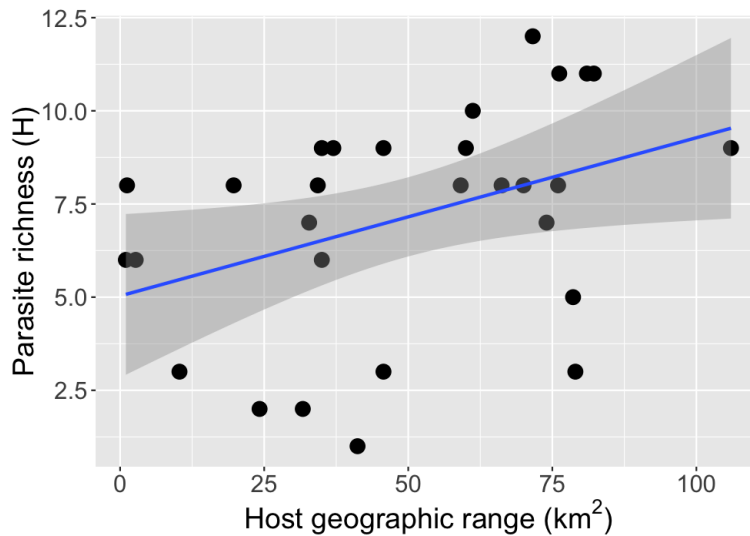


Figure 4. Linear regression between parasite diversity of butterflyfish species and host geographic range. Dark grey zone represents the 95% confidence interval.

3.3. Taxonomic composition of dactylogyrid assemblages

Thirteen dactylogyrid species were identified from the gills of the butterflyfish examined from the TIWP. Although dactylogyrid specificity varied between species, all species were found to be generalists among the Chaetodontidae. *Euhaliotrematoides triangulovagina* had the lowest specificity, being found on 29 host species, followed by *H. aurigae* and *E. grandis* (28 host species each). *Haliotrema scyphovagina* had the highest specificity, being found only on *C. unimaculatus* and *C. vagabundus* (Figure 5).

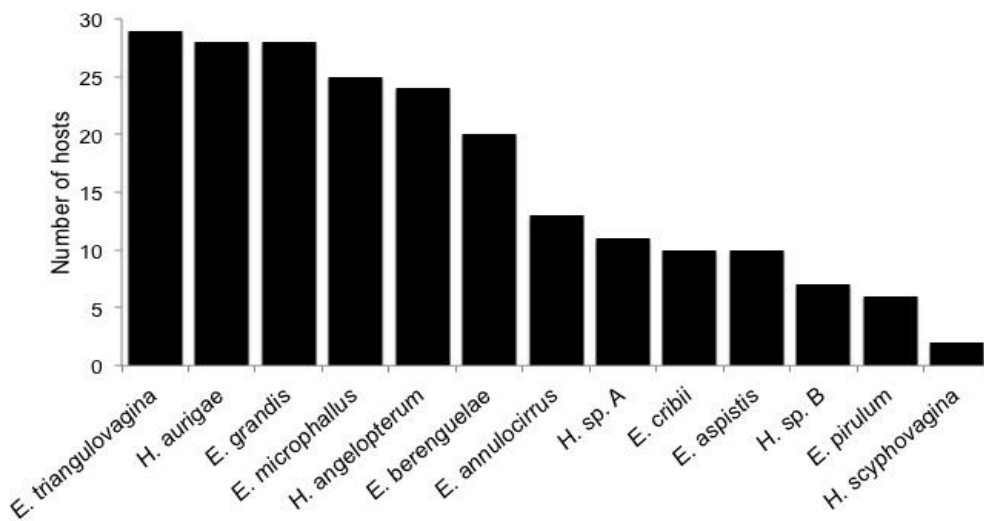


Figure 5. Host specificity for the 13 dactylogyrid species identified in this study.

Structure of the dactylogyrid communities was analyzed for each species of butterflyfish (Figure 6), and significant differences in the structure of communities were found between host species. Most fish species were parasitised by several dactylogyrid species, but relative abundances differed dramatically between host species. *Chaetodon semeion* was the only fish species parasitised by just one monogenean species (*H. aurigae*). *Chaetodon baronessa* also showed little parasite diversity, harbouring just two monogenean species (*H. aurigae* and *E. triangulovagina*) (Figure 6).

In order to analyse differences between the dactylogyrid communities for different fish species, a hierarchical cluster analysis was performed. Six groups based on monogenean community similarity were recognised (Figure 7). *Heniochus varius* Cuvier, 1829, whose dactylogyrid community was dominated by *E. microphallus*, had the most distinctive community structure, followed by *H. polylepis*, whose dactylogyrid community was mainly constituted by *E. triangulovagina*. The third group to diverge from the rest of the fish species was constituted by *C. quadrimaculatus*, *C. mertensii* Cuvier, 1831, *C. trichrous* Günther, 1874, *C. unimaculatus*, *C. reticulatus* and *C. ornatissimus* and was characterised by a high dominance of *H. angelopterum*. The fourth group comprised only *H. acuminatus* and *C. marginalis* with dactylogyrid communities dominated by *E. berenguelae*. The two closest cluster groups (fifth and sixth) had communities with a considerable presence of *H. aurigae*.

While the fifth group was constituted by fish species with dactylogyrid communities highly dominated by *H. aurigae*, the sixth group communities were more diversified (Figure 6 and 7). The parasite correlation circle showed that there were no marked negative correlations between parasite species, however some parasite species had positive correlations such as *H. aurigae*, *E. pirulum* and *E. grandis* and *H. angelopterum* and *E. microphallus* (Fig. 8).

Results from multivariate regression on distance matrices (MRM) showed that host phylogenetic similarities was the only significant factor ($P = 0.003$) explaining different taxonomic composition of dactylogyrid assemblages in butterflyfishes, while host ecology and geographic distributions were non significant ($P > 0.05$). However, the regression coefficient obtained for the model was extremely low ($R^2 = 0.018$).

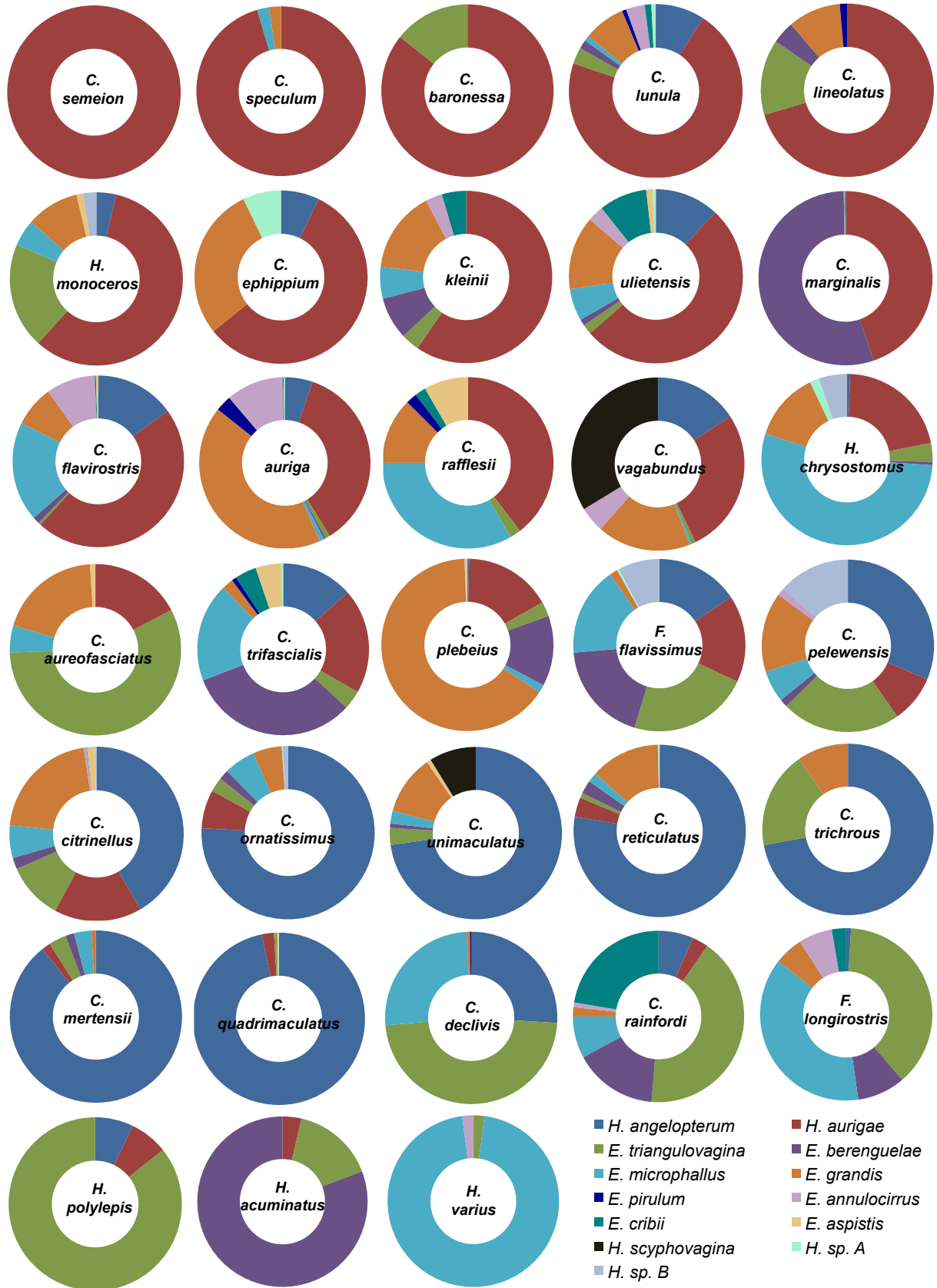


Figure 6. Dactylogyrid community structure for the butterflyfish species studied in the TIWP.

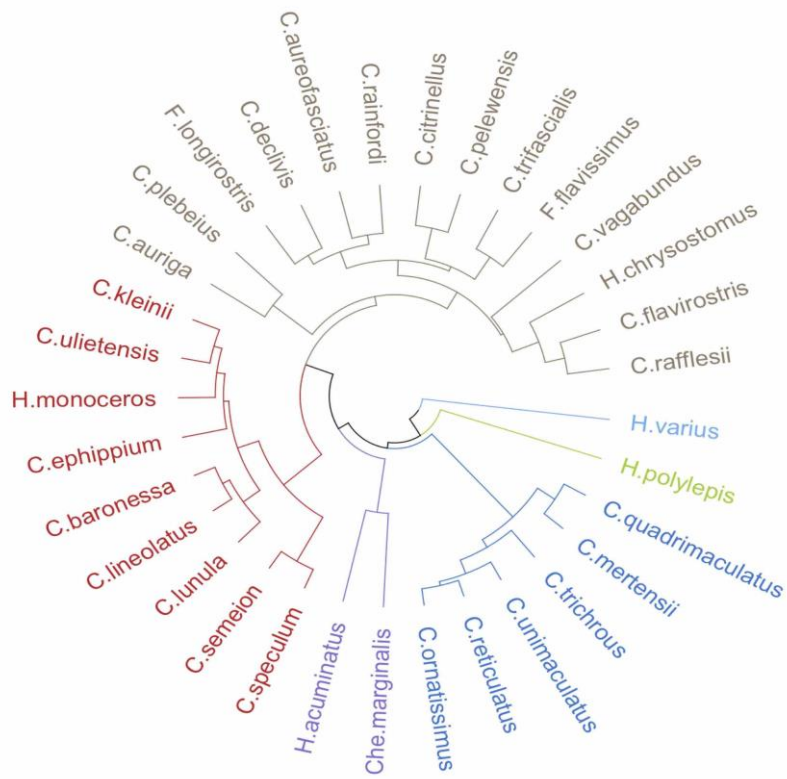


Figure 7. Cluster dendrogram of butterflyfish species based on their dactylogyrid community similarities. Colors show the sub-cluster identified with the KGS penalty function and the cutree function for R.

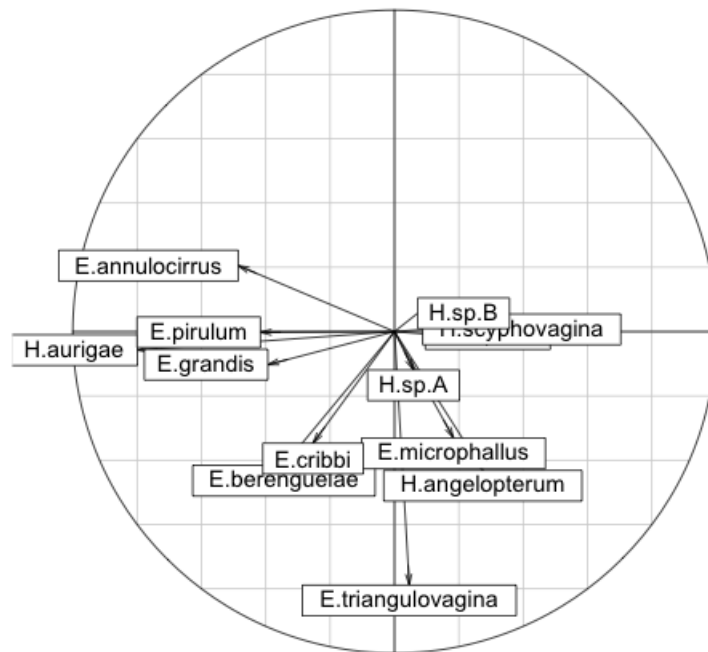


Figure 8. Correlation circle between the dactylogyrid species identified in this study.

4. Discussion

4.1. Dactylogyrid intensity, prevalence, richness and diversity

Since parasite communities are influenced by host physiology, ecology and phylogeny, phylogenetically close host species with similar ecologies can be expected to be infected by similar parasite communities (Muñoz et al. 2006; Tavares and Luque 2008). In this study, the dactylogyrid communities of 34 butterflyfish species from tropical Indo-west Pacific were studied and results indicated that parasitism intensities between closely related sympatric fish species varied significantly. Dactylogyrid intensity differences were broadly independent of the host ecological factors studied (size, depth range, geographical range and feeding behaviour) as well as host phylogeny. Sikkel et al. (2009) also found significant differences in intensity and prevalence of *Neobenedenia melleni* (McCallum, 1927) (Monogenea: Capsalidae) on three species of sympatric surgeonfishes and hypothesised that those can arise from microhabitat differences or from species-specific chemical cues. Our results revealed that fifteen butterflyfish species were always parasitised by dactylogyrids whereas *C. lunulatus* was never parasitised.

The ecology and phylogenetic position of *C. lunulatus* has been extensively studied (Pratchett, 2005; Pratchett et al. 2006a; 2014, Fessler and Westneat, 2007, Hsu et al. 2007) and there is no obvious peculiarity that could explain this marked difference in parasitism. For example, *C. lunulatus* and *C. ornatissimus* are ecologically and phylogenetically close, both species being obligate corallivores and occurring in coral-rich areas of lagoons (Hsu et al. 2007; Berumen et al. 2011). However, unlike *C. lunulatus*, *C. ornatissimus* is always parasitised by dactylogyrids. In further contrast, *C. lunulatus* has a relatively rich fauna of skin and gut trematodes most of which are also found in *C. ornatissimus*, with two species of faustulids (*Paradiscogaster oxleyi* Díaz, Bray & Cribb, 2013 and *Paradiscogaster flindersi* Bray, Cribb & Barker, 1994), one monorchiid (*Hurleytrematoides dollfusi* McNamara et al. 2012), one lecithasterid (*Aponurus chelebesoi* Bray & Cribb, 2000) and the blood fluke *Cardicola chaetodontis* Yamaguti, 1970, reported in previous studies (Bray & Cribb, 2000; McNamara and Cribb, 2011; McNamara et al. 2012; Díaz et al. 2013; Yong et al. 2013). It seems that the differences in monogenean intensities between closely related species such as *C. lunulatus* and *C. ornatissimus* must therefore arise from gill or gill mucus species-specific

characteristics. Some studies (Buchmann, 1999; Buchmann and Lindenstrøm, 2002) have analysed monogenean-host interactions and have found that immune mechanisms in fish skin and mucus play a role in monogenean specificity. Chemical composition of fish skin and mucus seems to be species-specific and studies, like those of Yoshinaga et al. (2002) and Ohashi et al. (2007), have identified some glycoproteins in fish mucus that induce oncomiracidia attachment in two species of capsalids. Recently, numerous studies have focused on fish mucus molecules and bioactivities and an antimicrobial peptide derived from the catfish gill hemoglobin was found to display potent antiparasitic activity against the ciliate parasites *Ichthyophthirius multifiliis* (Fouquet) (Ullal et al; 2008; Gómez et al. 2013; Rakers et al. 2013).

Skin and mucus microbiome are also species-specific and some recent studies showed that mucus commensal microbiota play a role in providing protection against opportunistic infections (Larsen et al. 2013; Boutin et al. 2014). Lowrey et al. (2015) found antifungal activity in bacteria isolates from skin of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) and Sánchez et al. (2012) found that bacteria from fish intestinal mucus produced a novel bioactive lipid sebastanoic acid with antibacterial activities. We thus suggest that studying *C. lunulatus* gill mucus molecules and microbiome could be highly interesting in order to investigate whether this species possesses some chemical or microbiota particularities that could explain the absence of dactylogyrid parasites. *C. lunulatus* and their sister species *C. trifasciatus* Park 1797 (Indian Ocean) and *C. austriacus* Rüpell, 1836 (Red Sea) are reported to be among the most dominant species in coral reefs worldwide, highlighting the functional importance of these butterflyfish species in coral reefs (Findley and Findley, 2001). Analysis of the dactylogyrid community infecting the sister species of *C. lunulatus*, might also give some insights into the mechanisms of parasite evasion in *C. lunulatus* and maybe clarify whether the absence of some parasitic groups such as dactylogyrids in *C. lunulatus* can be related to its demographic success over its congeners.

Kearn (1994) proposed that direct life cycles and the absence of specialised transmission stages in monogenean parasites could favour host switching. This hypothesis would predict that closely related sympatric host species would have similar parasite richness and diversity. However, dactylogyrid richness and diversity in sympatric butterflyfish species was highly variable in this study. For instance, *C. auriga* (host to 11 dactylogyrid spp.) and *C. ephippium* (host to 4 dactylogyrid spp.) inhabit similar ecological niches but exhibit very different

parasite richness. *C. quadrimaculatus* (host to 6 dactylogyrid spp., $H = 0.17$) and *F. flavissimus* (host to 8 dactylogyrid spp., $H = 1.82$) are also frequently found in the same habitats but have dramatically different monogenean diversity, indicating the possible presence of species specificities.

Several studies have shown that larger hosts can sustain greater number of parasites and may be expected to harbor richer parasite communities (Sasal et al. 1997; Sasal and Morand, 1998). In our study, no correlation was found between dactylogyrid richness and host size, and one of the smallest butterflyfish species (*C. citrinellus*) possessed the highest parasite richness. Morand et al. (2000) also failed to find a relationship between endoparasite communities and host size of butterflyfish species in New Caledonia. Host diet is also known to be an important parameter in parasite richness, especially in parasites with complex life cycles where hosts with broader diet ranges could acquire more trophically transmitted parasites than those with narrow, specialised diets (Simkova et al. 2001; Cirtwill et al. 2016). Such is the case for endoparasites in butterflyfish where host diet was a good predictor of parasite richness (Morand et al. 2000). A recent study has showed that host diet affected ectoparasite communities in bats due to different host fitness directly related to host diet (Luguterah and Lawer, 2015). In our study we did not find any correlation between parasite intensity or richness and feeding specificity, since one of the species with the most diverse monogenean communities, *C. trifascialis*, is among the most specialised, feeding almost exclusively on *Acropora hyacinthus* (Pratchett, 2005). Some authors have proposed that hosts with a larger depth range would encounter more parasite species and therefore have richer parasite faunas (Luque et al. 2004), however this was not the case for the dactylogyrids communities. Although some authors disagree, host species with large geographical ranges could have greater chances of acquiring new parasite species via colonisation (Simkova et al. 2001; Poulin, 2005a). In this study, the positive relationship between host geographic range and parasite species richness seemed to explain some of the observed parasite richness variability on different butterflyfish species, but the effect was moderate. Finally, the role of host phylogeny in the diversity and structure of parasites communities has been discussed extensively. While some studies like those of Morand et al. (2000) and Poulin et al. (2011) confirmed that host phylogenetic relationships have a strong influence on patterns of parasite richness, other provide evidence that host phylogeny might have only a weak influence on parasite richness and diversity (Muñoz et al. 2006; Alarcos and Timi, 2012). In our study,

host phylogeny was not related to parasite species richness. However, we should highlight that this is one of the first studies where quite homogeneous parasite communities (13 species of parasites belonging to the same family) were assessed in a highly diversified host family. McNamara et al. (2012) studied the monorchiid communities from chaetodontids and their results also show a weak influence of chaetodontids phylogeny on prevalence and richness of monorchiids. Yong et al. (2013) found that prevalence of the blood fluke *C. chaetodontis* varied importantly among closely related chaetodontids.

4.2. Taxonomic composition of dactylogyrid assemblages

Monogeneans are generally considered to be highly host-specific (Whittington et al. 2000). In this study we found just one species of dactylogyrid (*H. scyphovagina*) to be relatively host-specific and that most species parasitise a high number of chaetodontid species. Significant differences between taxonomic composition of dactylogyrid assemblages of sympatric host species were found, but no relationship was found between dactylogyrid community structure and host ecological factors and host geographic distribution. Only a very weak relationship was found between dactylogyrid taxonomic composition and host phylogeny, indicating the presence of other species-specific factors driving structure of dactylogyrid communities. *C. semeion* was uniquely parasitised only by *H. aurigae*, despite this species being found living among other fish species that harboured more dactylogyrid species. Although six dactylogyrid species were found infecting *C. quadrimaculatus*, over 95% of the dactylogyrid assemblage was constituted of *H. angelopterum*. Relative abundances of parasite species varied among chaetodontid species and we identified 6 groups of species that were dominated by different dactylogyrid species. The majority of the species (cluster 5 and most of cluster 6) were characterised by a dominance of *H. aurigae*, with cluster 5 having very high abundances of *H. aurigae*. The second most abundant dactylogyrid on butterflyfish was *H. angelopterum*, which clearly dominated monogenean assemblages of six fish species. *H. aurigae* and *H. angelopterum* are two of the largest dactylogyrid species studied and thus we suspect that, when in competition, they may appropriate most of the available space. *H. varius*, which constituted a group by itself, had the most distinct dactylogyrid community, dominated by *E. microphallus*; however, neither host ecology or phylogeny explains this marked divergence. *H. polylepis* had the second most different

parasite assemblage. This species possesses a very particular ecology, being the only species in the analysis always found on outer-shelf reefs and feeding on plankton which could explain different dactylogyrid assemblages (Emslie et al. 2010). *H. acuminatus* is also a planktivorous fish that inhabits unusually deep waters, which might partly explain its distinct parasite community, but little is known about the ecology of the Australian *C. marginalis* that could explain the similarity of its dactylogyrid community to that of *H. acuminatus*.

Since little is known about the ecology of butterflyfish dactylogyrids, their community assemblages could be partly determined by inter-parasite interactions on their hosts as shown in some other systems (e.g. Hughes and Boomsma, 2004). We did not observe strong negative correlations between dactylogyrid species, but however positively related species could occupy different gill microhabitats in order to avoid direct competition as proposed by Soler-Jiménez and Fajer-Ávila (2012) in dactylogyrids from wild spotted snapper (*Lutjanus guttatus*, Steindachner, 1868). However, to better understand dactylogyrid interaction with and within the host, more studies should be conducted on parasite microhabitats.

In summary, the gill monogenean communities of 34 sympatric butterflyfish species differ considerably in terms of intensities and in the structure of the dactylogyrid assemblages. One otherwise unremarkable species, *C. lunulatus*, was never parasitised, suggesting the involvement of gill species-specific parameters such as chemical cues. Dactylogyrid richness among butterflyfish species is not detectably related to host size, diet, depth range or phylogeny. However, there was a positive (although not strong) correlation between monogenean richness and host geographical range. Finally, we were able to identify six distinct groups of butterflyfishes based on the similarity of their dactylogyrid communities. Dactylogyrid communities in chaetodontids are evidently extremely complex, and although some differences between fish species could be explained by host geographical range, most of the drivers remain unclear. This suggests that other species-specific parameters or within-host interactions have major roles in shaping the dactylogyrid communities of butterflyfish.

SUPPLEMENTARY MATERIAL

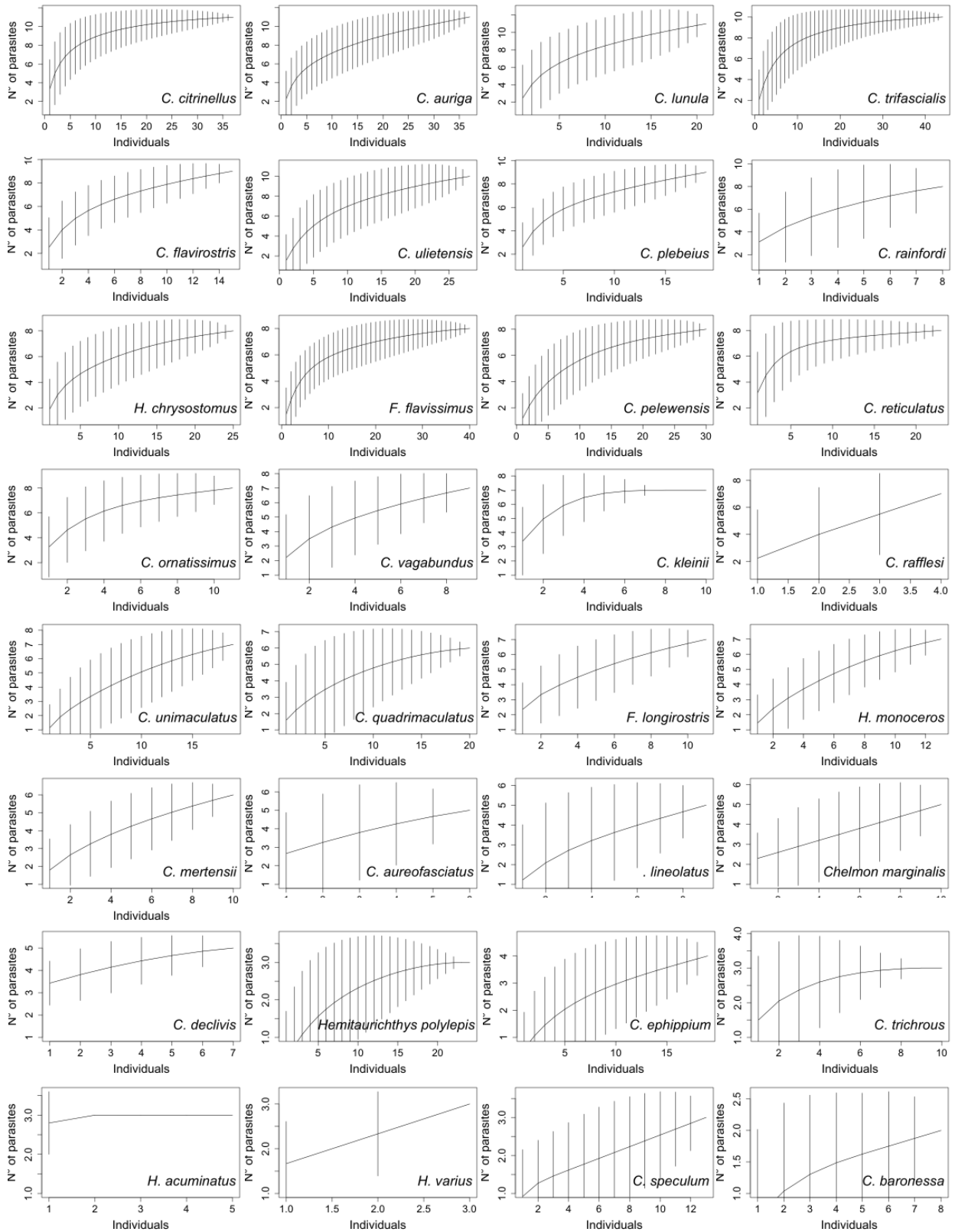


Figure S1. Accumulation curves for the 33 parasitised butterflyfish species.



CHAPTER II

Biogeography of gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfish from tropical Indo-west Pacific Islands

Reverter, M., Cribb, T.H., Cutmore, S.C., Bray, R., Parravicini, V., Sasal, P. Gill monogenean communities (Platyhelminthes, Monogenea, Dactylogyridae) of butterflyfishes from tropical Indo-West Pacific Islands. *Parasitology*. To be submitted to Journal of Biogeography (IF = 3.9)

Biogeography of coral reef fish ectoparasites in the West Indo-Pacific: how biogeographical processes shaped current host-parasite specificities in butterflyfish dactylogyrids (Monogenea).

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Abstract

Aim Investigate the biogeography of dactylogyrid parasites from butterflyfishes in order to better understand the spatial structure of coral reef fish ectoparasites in the West Indo-Pacific.

Location West Indo-Pacific Ocean.

Methods Dactylogyrid parasites were dissected from gills of 34 butterflyfish species (n = 560) collected at 9 localities. Prevalence and mean intensity of dactylogyrid species at each location and their area of distribution were determined. Pearson correlation was computed between dactylogyrid species prevalences. Relative abundance of dactylogyrid species, weighted parasitism intensity, diversity and richness were calculated for each locality. A hierarchical cluster analysis allowed identifying geographic regions with similar monogenean assemblages. Multiple regressions on distance matrices were used to study the contribution of sampled fish species, geographic distance and environmental differences between localities on parasite community dissimilarity (turnover, nestedness and Bray-Curtis dissimilarity).

Results Butterflyfish dactylogyrids are widely distributed throughout the West Indo-Pacific. Dactylogyrid assemblages are dominated by the *Haliotrema* species *H. angelopterum* and *H. aurigae*, which are negatively correlated between them ($\rho = -0.59$). Parasite richness and diversity are the highest in French Polynesia and the Great Barrier Reef. We identified three biogeographic regions based on dactylogyrid dissimilarities: French Polynesia, characterized

by high abundances of *H. angelopterum*, the western Indo-Pacific containing Lizard Island, Palau and Wallis, which exhibited high abundances of *H. aurigae* and Ningaloo reef, which presented high abundances of *Euryhaliotrema berenguelae* (48%). Differences between *Chaetodon* assemblages are the main factor driving parasite community turnover, whereas environmental dissimilarity was the most important factor explaining nestedness of parasite communities.

Main conclusions Our results support the presence of two different biodiversity hotspots of dactylogyrids in the Indo-West Pacific: the southwest Pacific and French Polynesia, where a turnover of the main *Haliotrema* species might indicate past biogeographical episodes which might have shaped host-parasite specificities.

Key words: biogeography, dactylogyrids, butterflyfish, host specificity, turnover, nestedness.

1. Introduction

Parasitism is considered to be the most common lifestyle on earth (Lafferty et al. 2008). Parasite impact on host fitness and consequent modification of competitive and trophic interactions among species have profound effects on the community structure and population dynamics (Hudson et al. 2006). Healthy ecosystems are reported to harbor a high diversity of parasites, however, current scenarios of environmental change, biological invasions and emerging diseases are likely to have numerous impacts on parasitic populations (Perkins, 2010; McLeod and Poulin, 2012; Altizer et al. 2013). Lafferty et al. (2008) showed that fish parasite richness was higher in a well-conserved atoll than in a heavily fished island, indicating the effect of some ecosystem disturbances on the parasite communities of coral reef fish. Therefore, understanding the parasite distribution, biogeography and the relationship between environmental variables and the parasitic communities is highly important in studies on coral reef resilience (Sikkel et al. 2009).

Biogeography of free-living organisms such as fish, has been studied consistently and nowadays there are several universal biogeographical theories that explain their patterns of biodiversity at large spatial and temporal scales (Floeter et al. 2007; Briggs and Bowen, 2013; Kulbicki et al. 2013). For example, the latitudinal diversity gradient (LDG) which states that diversity increases with latitude decrease or the distance decay in similarity that proposes that similarity in biological communities decreases with increasing biogeographical distance between them (Gaston 2000; Soininen et al. 2007). However, biogeography of parasites, especially of coral reef fish parasites remains poorly studied and elusive. Since parasites are strictly dependent on their hosts for essential resources (nutrients and physical attachment), their geographical distribution, biodiversity and evolutionary history is closely related to host communities (Poulin et al. 2011b). Besides, in host-parasite interactions, the evolution of parasite-avoidance strategies by hosts and parasite counter-adaptations to overcome host defence mechanisms leads to a tight host-parasite coevolution and parasite specificity (Klein et al. 2004). Past geological episodes such as geographic isolation between host communities could have been a major determinant in the host-parasite coevolution and current parasite specificity (Poulin et al. 2011a; Martinez-Aquino et al. 2014). Therefore, the study of the biogeography of parasite communities coupled to the study of parasite specificities among

multiple hosts can provide insights into the differential evolution of parasitic communities over spatial scales and help us better understand parasite-host interactions.

Beta-diversity was first defined by Whittaker (1960) as the extent of change in community composition among sites. Two different phenomena can produce differences in species compositions between two sites: spatial turnover and nestedness (Baselga, 2010). Spatial turnover is the replacement of some species by others from site to site, while nestedness of species assemblages occurs when the community of a poorer site is a subset of a richer site (Baselga 2010; 2012). The differentiation of the spatial turnover and nestedness components of community dissimilarity is crucial to understand the biogeographic patterns of species, since they can be generated from different underlying processes (Williams et al. 1999). For example, spatial turnover patterns suggest the existence of spatial and historical constraints, whereas nestedness reflects a non-random process of species loss (Baselga, 2010).

Butterflyfishes (Chaetodontidae) are a diverse and emblematic family of coral reef fishes. They are among the most broadly distributed reef fishes, and their ecologies and distribution patterns have been studied consistently (Pratchett et al. 2013; diBattista et al. 2015). They, thus, present an outstanding system to study spatial distribution and biogeography of parasites. Butterflyfishes are parasitized by gill monogeneans belonging to the family Dactylogyridae, with a total of 15 species reported from chaetodontids to date (Plaisance and Kritsky, 2004; Plaisance et al. 2004). Dactylogyrids are the primary monogenean group found on this host family; a few capsalids are the only other monogeneans known to infect chaetodontids (Bullard et al. 2000). Dactylogyrids are small flatworms (ranging from 160 to 490 μm in length) that attach to the gills where they feed and reproduce sexually (Plaisance et al. 2004; Plaisance and Kritsky, 2004). Their eggs leave the host and hatch in the water to release a swimming larva (oncomiracidia) that must find a new host within 12-24 hours (Gannicott and Tinsley, 1998).

The aim of this study is to investigate the biogeography of dactylogyrid parasites from butterflyfishes in order to better understand the spatial structure of coral reef fish ectoparasites in the West Indo-Pacific and to investigate if biogeography is related to the observed dactylogyrid specificity among butterflyfishes (Reverter et al. 2016). Since the biogeography of dactylogyrid parasites from butterflyfish has been little studied, we first determined the

area of distribution, local prevalences and abundances for thirteen dactylogyrid species. We then studied the structure of the dactylogyrid communities at nine geographical locations, and assessed the effect of geographic distance, similarity in host communities and environmental similarities on both components of parasite community dissimilarity: species turnover and community nestedness.

2. Materials and methods

2.1 Sampling

Thirty-four species of butterflyfish ($n = 560$) were collected at 8 localities in the Pacific: Palau, Wallis and Futuna, Lizard Island (Australia), and the 5 archipelagos in French Polynesia (Society Islands, Tuamotu Islands, Austral Islands, Gambiers Islands and Marquesas Islands). Butterflyfish were also sampled from a single locality in the Indian Ocean, from Ningaloo Reef on the west Australian coast (Figure 1).

Adult butterflyfish were captured, put in individual plastic bags with seawater and brought immediately to a laboratory, where gills were dissected and fixed in 70% ethanol. Dactylogyrids were removed from the gills using a stereo microscope and were mounted on glass slides in Malmberg's ammonium picrate-glycerine.

All dactylogyrids from each individual fish were observed under a compound microscope, and parasite species and number were recorded. Dactylogyrid species determination was according to published literature (Plaisance and Kritsky, 2004, Plaisance et al. 2004; Kritsky, 2012) and was based on the morphological characters of the sclerotised structures (the haptor and the reproductive organs).

Sampling was performed in order to be as representative as possible of the butterflyfish community for each of the localities, so differential sampling of butterflyfish species reflects changes in butterflyfish communities between localities.

2. 2 Data analysis

Dactylogyrid prevalence (% of parasitized fish) and mean intensity (number of parasites in parasitized fish) were determined for each dactylogyrid species at each geographic site. Dactylogyrid areas of distribution were determined using presence-absence of the dactylogyrid species at the 9 studied geographic locations, combined with previous records found in the literature (Yamaguti, 1968; Dyer et al., 1989; Zhang et al. 2001; Plaisance and Kritsky 2004, Plaisance et al. 2004). Pearson correlation was used to test correlations between dactylogyrid species prevalences.

Parasite communities were studied at each geographic site, and the relative abundance of dactylogyrid species (contribution of a dactylogyrid species to the overall dactylogyrid community expressed in %) and the weighted parasitism intensity were calculated. The weighted parasitism intensity at each geographic site ($I_{w_{site}}$) was computed in order to correct the effect of differential parasitism intensities from different butterflyfish species sampled at different geographic locations (Reverter et al. 2016). Weighted parasitism (I_w) intensity was calculated according to the following equation:

$$I_{w_{site}} = \frac{\sum_{sp1}^{spx} (I_{sp_{site}}/I_{sp})}{n^{\circ} sp_{site}}$$

where $I_{sp_{site}}$ is the mean parasitism intensity of a fish species ($sp_{1,2,x}$) at one geographic site, I_{sp} is the global mean parasitism intensity of that fish species and $n^{\circ} sp_{site}$ is the number of fish species sampled at that geographic site.

Parasite diversity at each geographic site was computed using the Shannon diversity index (H) and parasite richness was also calculated. A hierarchical cluster analysis («hclust» package for R) using the Bray-Curtis distance was used to identify geographic regions with similar monogenean assemblages. The "Average" algorithm was chosen after analysis of the cophenetic correlation coefficients (Pearson correlation between the cophenetic distances calculated on cluster branches and the parasite dissimilarity matrix) (Kulbicki et al. 2013). The Kelley-Gardner-Sutcliffe (KGS) penalty function was used to prune the dendrogram (Bottegoni et al. 2006).

The function "bray.part" from the package betapart (R Software) was used to obtain 3 distance matrices of the parasite communities at different locations, accounting for the balanced variation (species turnover, dBC-bal) and the abundance gradient (species nestedness, dBC-gra) components of Bray-Curtis dissimilarity and the sum of both values (Bray-Curtis dissimilarity, dBC) (Baselga, 2013). Multiple regression on distance matrices (MRM, package ecodist for R, 1000 permutations) was used to study the contribution of sampled fish species, geographic distance and environmental differences between localities on dissimilarity in parasite communities (turnover and nestedness and Bray-Curtis dissimilarity) (Lichstein, 2007). Linear geographic distances between pairs of geographic locations were obtained. The environmental parameters considered in the MRM analyses were chlorophyll concentration, cloud cover, par radiation, dissolved oxygen, nitrate concentration, phosphate concentration, silicate concentration, salinity and sea surface temperature. Distance-decay curves were generated for factors that had a significant effect in the dissimilarity of parasite communities (*Chaetodon* species and environmental conditions).

3. Results

3.1. Dactylogyrid area of distribution

Dactylogyrid parasites from butterflyfishes are widely distributed throughout the Indo-West Pacific region, from Ningaloo Reef in the Indian Ocean and the South China Sea to the most western Polynesian archipelago, the Gambiers (Figure 1). Most dactylogyrid species were found at all the sampled locations, and the absence of some rare dactylogyrid species in some locations (prevalence lower than 20% in all the locations) are likely explained by their low prevalence and sampling difficulty (Supp. Material, Table 1). For example, the absence of *Euryhaliotrema annulocirrus* and *E. aspistis* in samples from Wallis could be explained by their low prevalence rather than an actual absence and consequent fragmentation in their range between Australia and French Polynesia. Although the different dactylogyrid species share their habitat, their prevalences and intensities vary at different locations. Thus, *Haliotrema angelopterum* has its highest prevalences and abundances in French Polynesia, whereas the highest prevalences of *H. aurigae* are found at Ningaloo Reef (80%) and Lizard

Island (57%). *Euryhaliotrema berenguelae* is a relatively rare species at most sites (total prevalence < 15%), however 73% of fish in Ningaloo Reef were parasitized by high numbers of *E. berenguelae*. *Euryhaliotrema grandis* is most prevalent in the Tuamotu (48%), however the highest intensities were observed in fish from Ningaloo Reef. *Euryhaliotrema microphallus* is most prevalent in fish from Lizard Island (44%) (Figure 1). Pearson correlation was used to study the interspecific interactions between the different parasite species and species turnover. Results showed that *H. angeloferum* is negatively correlated with *H. aurigae* (-0.59, p-value < 0.05) and *E. cribbi* (-0.69, p-value < 0.05), and *H. aurigae* is positively correlated with *E. berenguelae* (0.79, p-value < 0.05) and *E. grandis* (0.63, p-value < 0.05). *E. microphallus* is also strongly correlated with *E. annulocirrus* (0.73) (Figure 2).

Dactylogyrid species	NR	LI	Palau	Wallis	Society	Tuamotu	Australis	Gambiers	Marquesas	HI	NC	Hawaii	SCS
<i>H. angeloferum</i>	56	117	5	1	854	764	1970	182	526	-	-	X	-
<i>H. aurigae</i>	487	469	81	53	134	593	459	127	46	X	X	X	-
<i>E. triangulovagina</i>	102	194	14	35	16	114	92	12	407	-	-	X	X
<i>E. grandis</i>	629	190	9	8	205	621	64	49	43	X	X	X	-
<i>E. berenguelae</i>	1173	94	2	11	10	21	77	37	-	-	-	-	-
<i>E. microphallus</i>	2	273	1	X	63	87	189	132	120	X	X	X	-
<i>E. annulocirrus</i>	-	20	X	-	17	4	48	27	-	X	X	X	X
<i>E. pirulum</i>	1	1	1	X	X	2	5	14	1	-	X	-	-
<i>E. cribbi</i>	-	79	5	30	4	3	6	1	-	-	-	-	-
<i>E. aspistis</i>	-	39	X	X	2	29	13	-	-	-	X	-	-
<i>H. sp. A</i>	-	11	-	-	-	-	92	-	-	-	-	-	-
<i>H. sp. B</i>	-	12	-	-	-	41	-	-	-	-	-	-	-
<i>H. scyphovagina</i>	-	-	-	-	-	23	-	-	-	-	-	X	-

Table 1. Abundance of dactylogyrid species at the nine sampled geographic sites. Presence (X) of dactylogyrid species obtained from literature. NR = Ningaloo Reef, LI = Lizard Island, HI = Heron Island, SCS = South China Sea.

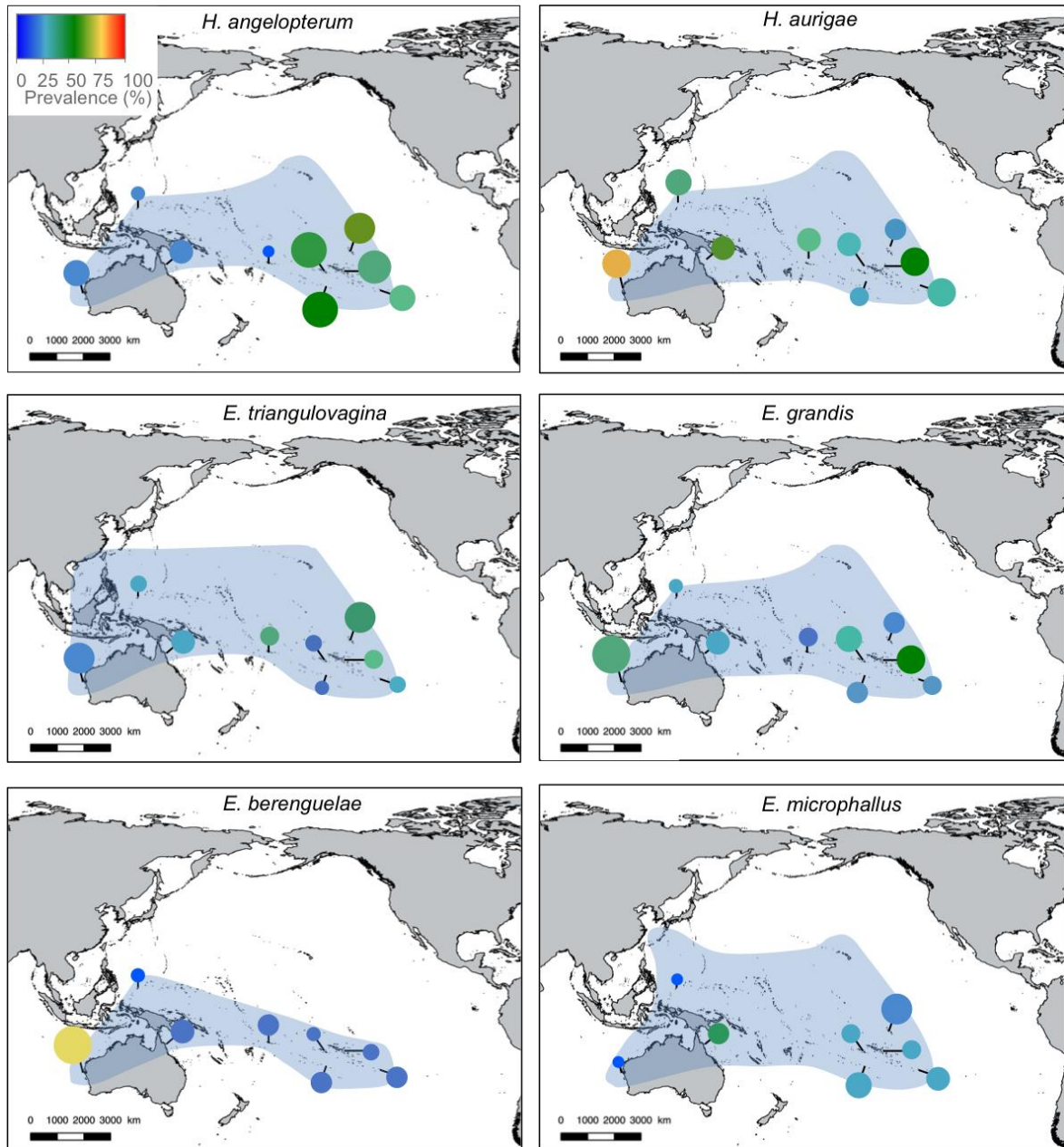


Figure 1. Areas of distribution (blue shadow), prevalence (circle colour) and abundance (circle size) of dactylogyrid parasites from butterflyfishes in the Indo-West Pacific Region. Only parasites with prevalences > 20% for at least one locality were plotted (other dactylogyrid species area of distribution are found in supplementary material).

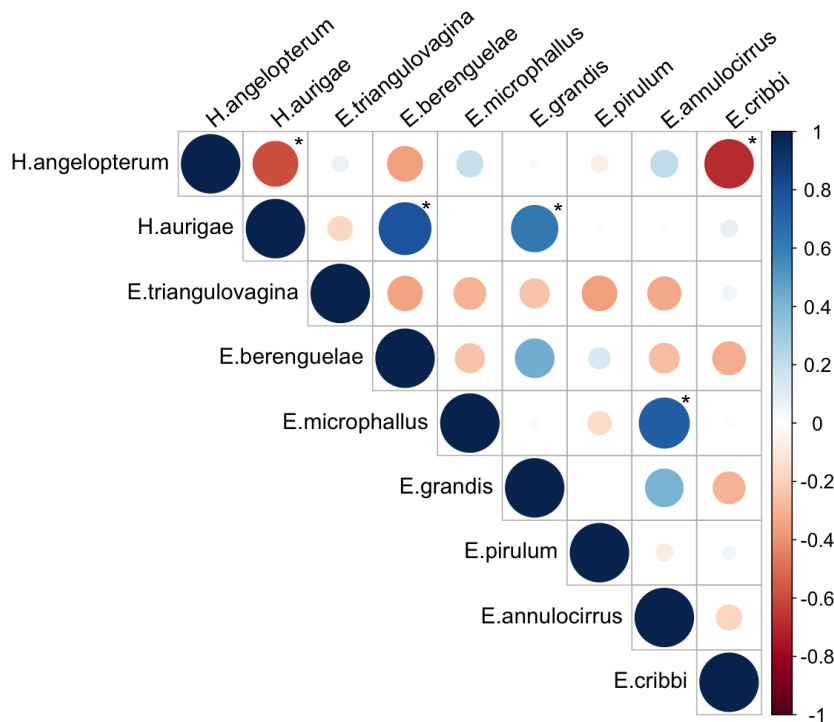


Figure 2. Correlation matrix between dactylogyrid species (Pearson correlation). Only dactylogyrid species found at least at 5 locations were plotted. * indicates p-value < 0.05.

3.2. Biogeographic structure of dactylogyrid communities

Weighted intensity, that is, dactylogyrid intensity where the effect of differential host species intensities has been corrected, showed striking differences between geographic locations. Fish from the Marquesas archipelago presented the lowest parasitism intensity ($I_{w\text{Marquesas}} = 0.57$), followed by Palau ($I_{w\text{Palau}} = 0.65$) and the Gambiers archipelago ($I_{w\text{Gambiers}} = 0.67$). Fish from the Tuamotu archipelago had the highest number of dactylogyrid parasites ($I_{w\text{Tuamotu}} = 1.49$) followed by the Society archipelago ($I_{w\text{Society}} = 1.12$) and Ningaloo Reef ($I_{w\text{NR}} = 1.09$) (Table 2, Figure 3).

Twelve out of the thirteen identified dactylogyrid species were found at the Tuamotu archipelago and Lizard Island (Table 1). Wallis and the Marquesas islands were the two locations where the lowest number of dactylogyrid species were found (6) (Table 2). Dactylogyrid diversity (H) was variable between geographic locations. Highest dactylogyrid diversity was found at Lizard Island (1.96) on the Great Barrier Reef, followed by the

Gambiers archipelago (1.74) and the Tuamotu (1.64). The lowest dactylogyrid diversity was observed in the Society archipelago (1.12), where the dactylogyrid community was strongly dominated by *H. angeloferum* (65%). Palau and the Marquesas islands also presented a notably low dactylogyrid diversity (1.22). Palau dactylogyrid assemblages were dominated mostly by *H. aurigae* (66%), while *H. angeloferum* (46%) and *E. triangulovagina* (36%) were the most abundant parasites in the Marquesas (Table 2, Figure 3).

Site	Richness	Shannon index (H)	Intensity	Weighted intensity
Society	9	1.12	111.3	1.12
Palau	8	1.22	24.4	0.65
Marquesas	6	1.22	60.7	0.57
Australs	11	1.24	79.8	0.99
Ningaloo Reef	7	1.25	132.4	1.09
Wallis	6	1.45	19.7	0.90
Tuamotu	12	1.65	126.5	1.49
Gambiers	9	1.74	26.5	0.67
Lizard Island	12	1.96	56.5	0.86

Table 2. Dactylogyrid diversity index (Shannon), richness, intensity and weighted intensity at the different geographic locations.

A hierarchical cluster analysis allowed identification of three main different biogeographic regions based on dissimilarities of their dactylogyrid communities, the eastern Pacific region containing French Polynesia, the western Indo-Pacific region containing Lizard Island, Palau and Wallis and Ningaloo Reef (Figure 4). French Polynesia was characterized by a high abundance of *H. angeloferum*, while localities in the western Indo-Pacific region exhibited higher abundances of *H. aurigae*. Ningaloo reef was highly dissimilar with a high abundance of *H. berenguelae* (48%) (Figure 3, 4).

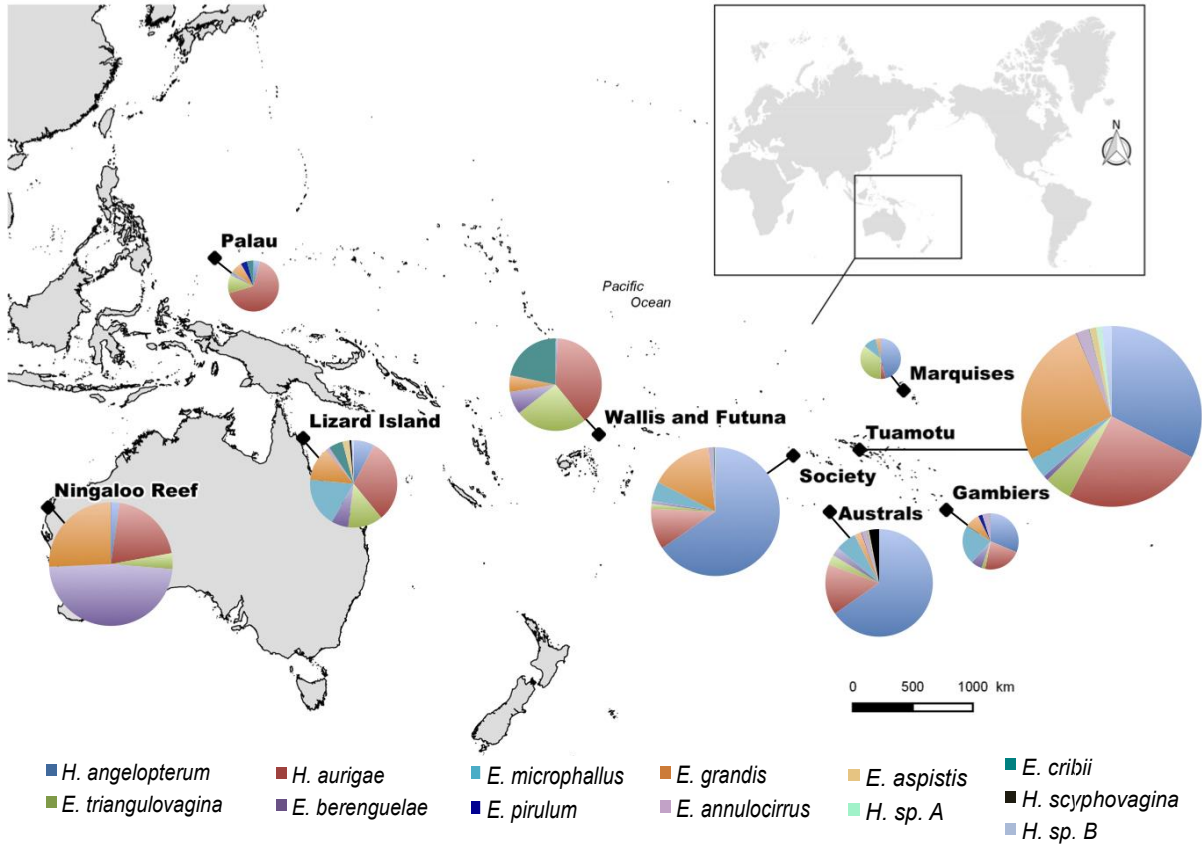


Figure 3. Structure of dactylogyrid communities at 9 locations in the West Indo-Pacific. Size of the circles is relative to the weighted intensity (Iw) for each location.

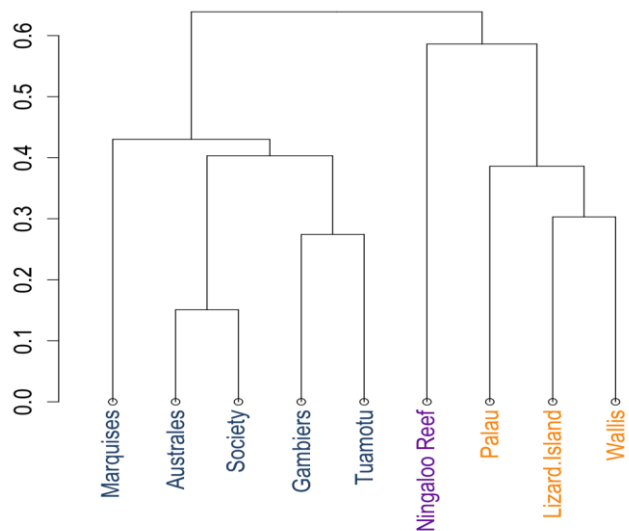


Figure 4. Cluster analysis of the parasite communities dissimilarities (Bray-Curtis distance, « average » algorithm).

3.3. Factors shaping biogeographic differences in dactylogyrid communities

Multiple regression models showed that most of the differences between dactylogyrid communities from geographic sites is due to differences in the butterflyfish communities (Table 2). Distance decay curve showed that similarity of dactylogyrid communities decreases with increasing butterflyfish dissimilarity ($R^2 = 0.64$) (Figure 5a). The *Chaetodon* species sampled at different locations (both balanced variation and abundance gradient) had a significant effect on the balanced variation (dBC-bal) of the dactylogyrid communities (Table 2). Distance decay curves showed that balanced variation similarity of dactylogyrid communities (dBC-bal) decreases with an increase in the dissimilarity of the butterflyfish communities (dBC-gra, $R^2 = 0.39$) (Figure 5b). Environmental parameters had a significant effect on the gradient variation (dBC-gra) of dactylogyrid communities and distance decay curve showed that the gradient variation similarity of dactylogyrid communities decreased with increasing environmental dissimilarity ($R^2 = 0.32$) (Table 2, Figure 5c). Linear distances between geographic locations did not have a significant effect on the dissimilarity (dBC-bal, dBC-gra and dBC) of the dactylogyrid communities between sites (Table 2).

Response or dependent variable	P-value	R2 model	p-values	F
Parasite community balanced variation (dBC-bal)				
<i>Model 1</i>				
Environmental parameters	0.066	0.39	0.005	6.88
Linear distance	0.277			
Chaetodon species (dBC-gra)	0.005			
<i>Model 2</i>				
Environmental parameters	0.254	0.35	0.004	5.74
Linear distance	0.065			
Chaetodon species (dBC-bal)	0.012			
<i>Model 3</i>				
Environmental parameters	0.196	0.23	0.06	3.25
Linear distance	0.090			
Chaetodon species (dBC)	0.131			
Parasite community gradient abundace (dBC-gra)				
<i>Model 1</i>				
Environmental parameters	0.008	0.31	0.028	4.89
Linear distance	0.486			
Chaetodon species (dBC-gra)	0.418			
<i>Model 2</i>				
Environmental parameters	0.001	0.49	0.003	10.37
Linear distance	0.335			
Chaetodon species (dBC-bal)	0.014			
<i>Model 3</i>				
Environmental parameters	0.025	0.32	0.037	4.93
Linear distance	0.089			
Chaetodon species (dBC)	0.322			
Parasite community dissimilarity (dBC)				
<i>Model 1</i>				
Environmental parameters	0.096	0.58	0.001	14.77
Linear distance	0.615			
Chaetodon species (dBC-gra)	0.038			
<i>Model 2</i>				
Environmental parameters	0.032	0.46	0.005	9.23
Linear distance	0.087			
Chaetodon species (dBC-bal)	0.881			
<i>Model 3</i>				
Environmental parameters	0.126	0.64	0.003	19.30
Linear distance	0.765			
Chaetodon species (dBC)	0.003			

Table 2. Results from the multiple regression models (MRM) between the parasite dissimilarity matrices (balanced variation - dBCbal and gradient abundance component dBC-gra and Bray-Curtis dissimilarity –dBC) and the environmental dissimilarity, geographic distance and dissimilarity in *Chaetodon* species.

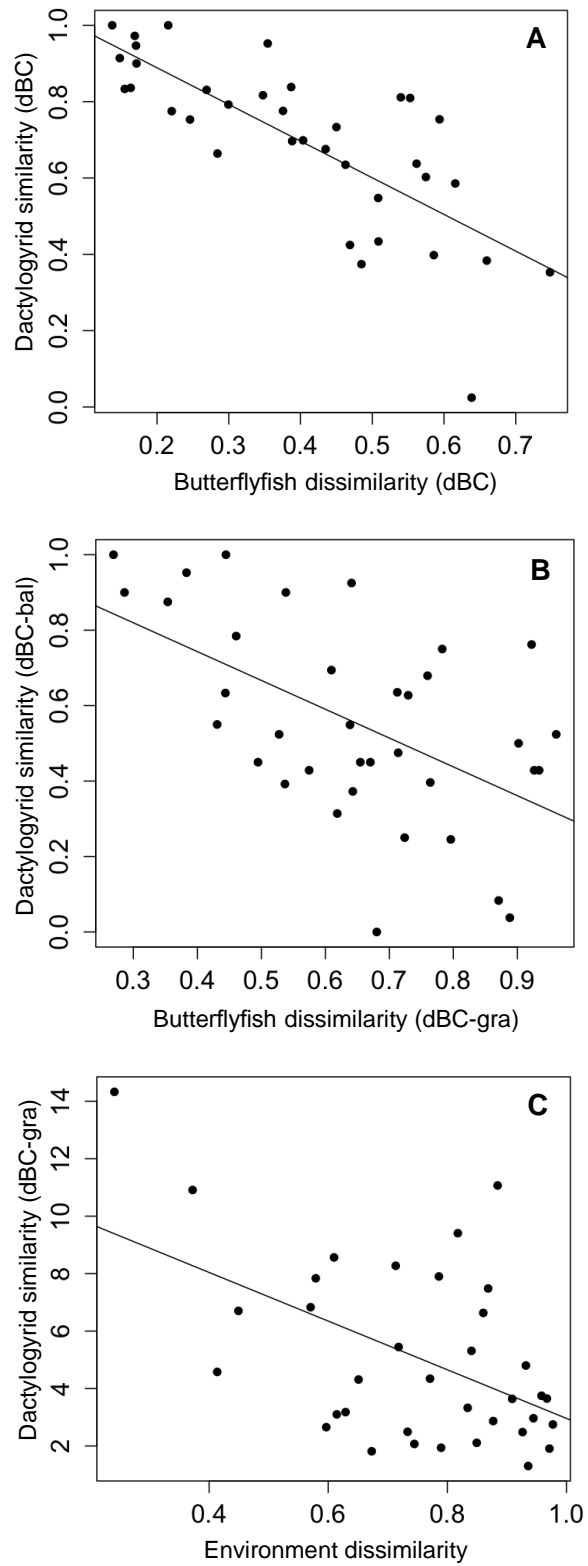


Figure 5. Distance decay curves for significant factors in parasite community dissimilarity.

Discussion

Spatial distribution of coral reef fish ectoparasites has been poorly studied. Butterflyfishes are among the most broadly distributed reef fishes, and their ecologies and distribution patterns have been studied consistently, thus they present an excellent opportunity to study spatial distribution and biogeography of their ectoparasites. Two studies investigated the biogeography of digenean parasites in several butterflyfishes from the Indo-West Pacific, and they found that French Polynesia presented a lower richness in species of *Transversotrema* and *Hurleytrematoides* (McNamara et al. 2012; Cribb et al. 2014).

Most dactylogyrid species from butterflyfishes were broadly distributed throughout the Pacific Ocean with the exception of some rare species that were absent at some locations. However, since prevalences of rare dactylogyrid species were, by definition, low, they could have been missed from our sampling. These findings could indicate that despite the possible distinct origins and evolutionary ages of the dactylogyrid species as proposed by Plaisance et al. (2008), these species have similar dispersal strategies that allow them to display similar geographic ranges. Our results show that butterflyfish monogenean assemblages are dominated by *Haliotrema* species (*H. angelopterum* and *H. aurigae*) throughout the whole studied geographical range. *H. angelopterum* highest prevalences and abundances are found in French Polynesia, whereas *H. aurigae* highest prevalences are found at Ningaloo Reef and Lizard Island. Correlation tests showed that these two species are negatively correlated, indicating a possible species turnover in the dactylogyrid community between French Polynesia and the western Indo-Pacific region.

The Coral Triangle that encompasses the Philippines, Indonesia and New Guinea is the area of highest marine biodiversity in the world, with marine richness radially decreasing from it (Bellwood and Hughes, 2001; Carpenter and Springer, 2005). For example, richness of shore fishes declines from 1693 at the center of the Coral Triangle in the Philippines to 1579 in the main island of New Caledonia, 702 fish species in the Society Islands and just 245 fish species in the Gambier Archipelago (Kulbicki, 2007). Biodiversity decrease of parasitic organisms with increasing distance from the Coral Triangle has been much less studied. However, a few works have shown a dramatic reduction of parasite richness from west to east Pacific (Rigby et al. 1997; McNamara et al. 2012; Cribb et al. 2014). In contrast, the few studies on butterflyfish dactylogyrids seemed to indicate greater parasite richness in French

Polynesia (Plaisance et al. 2004, Plaisance and Kritsky, 2004). Results from our larger study including 34 butterflyfishes and 13 monogenean parasites do not support a decrease in parasite richness or diversity from the Coral Triangle. Instead, we have discovered high parasite richness and diversity both in French Polynesia and the Great Barrier Reef. The lowest parasite richness in the Marquesas Islands is potentially explained by the isolation of this archipelago together with the absence of lagoons and very low coral cover and therefore a lower number of susceptible host species and number of individuals (Kulbicki, 2007). Bellwood and Hughes (2001) proposed that island size influenced local diversity, with smaller island supporting fewer species. Sampling in the Society archipelago was done in Scilly and Mopelia, two young small islands situated at the most western part of French Polynesia. Lower fish richness has been observed in those islands than in other Society Islands like Moorea, which is consistent with the lower dactylogyrid diversity observed there.

The cluster analysis showed that three main biogeographic regions can be recognised based on differences in the dactylogyrid communities of butterflyfishes: French Polynesia, the western Indo-Pacific (Palau, Lizard Island and Wallis) and Ningaloo Reef. Altogether our results indicate the possible presence of two different biodiversity hotspots of dactylogyrids in the Indo-West Pacific: the southwest Pacific and French Polynesia, supporting qualitatively and quantitatively different monogenean assemblages. Plaisance et al. (2008) studied the phylogeography of two dactylogyrid parasites from butterflyfishes and found clear genetic differentiation of *E. grandis* between the population in Moorea (French Polynesia) and all the other populations (West Pacific), suggesting a possible isolation of French Polynesia during Pleistocene sea-level fluctuations. These fluctuations might have been at the origin of the two biodiversity hotspots. Macpherson et al. (2010) also found different communities of galatheid squat lobsters between French Polynesia and the rest of the West Indo-Pacific region with a high proportion of endemism, also suggesting French Polynesia as a diversity center.

Distance decay in similarity, which states that similarity in species composition decreases with increasing distance between localities, is considered a universal pattern in ecology (Soininen et al. 2007; Morlon et al. 2008). Numerous studies have shown that similarity of parasite communities decreases with increasing distances (Oliva and Gonzalez, 2005; Pérez del Olmo et al. 2009; Thieltges et al. 2009; Timi et al 2010). However, some other studies have shown that other parameters such as host phylogenetic distance or environmental

conditions might play a more important role in similarity of parasite communities than geographical distance (Krasnov et al. 2005; Vinarski et al. 2007; Seifertova et al. 2008). To date, all the studies on parasite community similarities have been either on qualitative (i.e. species presence or absence) or quantitative data (i.e. species abundances). However, recent studies have shown that incidence-based measures of dissimilarities reflect two different phenomena, spatial species turnover, which is the species replacement, and nestedness of assemblages, which accounts for abundance gradients (Baselga, 2010; 2012). To the best of our knowledge, this is the first study on the biogeography of parasite communities that distinguished between the balanced variation (turnover) and abundance gradient (nestedness) components of dissimilarity in order to identify which parameters are driving each of the processes. Our results show that geographic distance between host populations was not a significant factor on either the turnover or nestedness of the parasite community. However, similarity of the host populations between sites was identified as the main driver responsible for the turnover of the parasite community and for the overall similarity in parasite communities. In a previous study, we showed that different butterflyfish species, even sympatric species, presented well delineated communities of dactylogyrids, and we identified 6 clusters containing species with similar dactylogyrid assemblages (Reverter et al. 2016) The present results reinforce the hypothesis of a strong specificity of dactylogyrid parasites, and the non-random organization of parasite communities within butterflyfish populations in the West Indo-Pacific. The two main clusters of butterflyfish species based on similarity of their dactylogyrid parasites were characterized by the dominance of *H. aurigae* and *H. angelopterum* respectively, with most species from cluster 1 primarily found in the western Indo-Pacific (e.g. *C. baronessa*, *C. speculum*, *C. semeion*, *C. lineolatus*) and species from cluster 2 most often found in French Polynesia (e.g. *C. reticulatus*, *C. trichrous*, *C. ornatissimus*) (Reverter et al. 2016). Prevalence differences (and a possible turnover) of these two dactylogyrid species, as discussed above, are among the main differences between the two geographic regions identified (French Polynesia and the western Indo-Pacific). *H. aurigae* and *H. angelopterum* are phylogenetically close, and they are among the biggest species of dactylogyrid parasites found in butterflyfishes (Plaisance et al. 2005). We could therefore imagine that the evolutionary history of these two parasite species, coupled to geological processes that determine their biogeography, have shaped parasite specificity over time through tight parasite-host coevolution. We suggest that *H. aurigae* and *H. angelopterum*

might have diverged from a common ancestor for example during French Polynesia Pleistocene isolation.

Recent studies have shown that environmental factors such as salinity or temperature are important factors in driving the composition of marine parasite communities (Thieltges et al. 2010; Poulin et al. 2011b; Blasco-Costa et al. 2015). In our study, environmental dissimilarity was the most important factor explaining nestedness of parasite communities. Pietroock and Marcogliese (2003) reported that free-living infective stages of helminth parasites can only survive and successfully infect a host within narrow and species-specific set of environmental conditions. Since chaetodontid larvae are not parasitized by monogenean parasites and dactylogyrid oncoramicidia can only live a few hours without a host, it has been proposed that dactylogyrid dispersal takes places through the passive dispersion of their eggs by oceanic currents (Ganicott and Tinsley, 1998; Plaisance et al. 2008). Several studies have shown that monogenean egg hatching is highly dependent on environmental parameters such as water temperature (Cecchini et al. 1998; Tubbs et al. 2005). Our results show that environmental parameters affect the gradient abundance of parasite species but not the parasite turnover, indicating the effect of environmental conditions on the suitability for egg hatching, larval development and successful infection of their hosts.

This study dramatically increases knowledge of dactylogyrid communities of butterflyfishes and their distribution in the West Indo-Pacific region. However, more studies should be conducted to determine the full distribution of these species, which might include other oceans (e.g. *E. annulocirrus* has been recorded in the Red Sea) (Paperna, 1972). We have observed two main biogeographic regions that are mainly differentiated by a turnover in the two major parasite species belonging to the genus *Haliotrema*. Differences between *Chaetodon* assemblages are the main factor influencing parasite community turnover, and coupled with the results from a previous work (Reverter et al. 2016), this suggests that biogeographical processes shaped parasite specificity probably through tight parasite-host coevolution. Environmental dissimilarity arose as the main factor in the nestedness of parasite communities, indicating the importance of favorable environmental condition for dactylogyrid egg hatching and effective host infection.

Finally, butterflyfishes are well known for their propensity to hybridize (Montanari et al. 2014), thus the study of dactylogyrid communities between hybrids and closely related

species from different oceans might provide helpful insights in their biogeography and evolutionary history. Similarly, the integrated study of the biogeography of direct cycle parasites (monogeneans) and complex cycle parasites (digeneans) might also provide new insights in the biogeography of coral reef parasites.

SUPPLEMENTARY INFORMATION

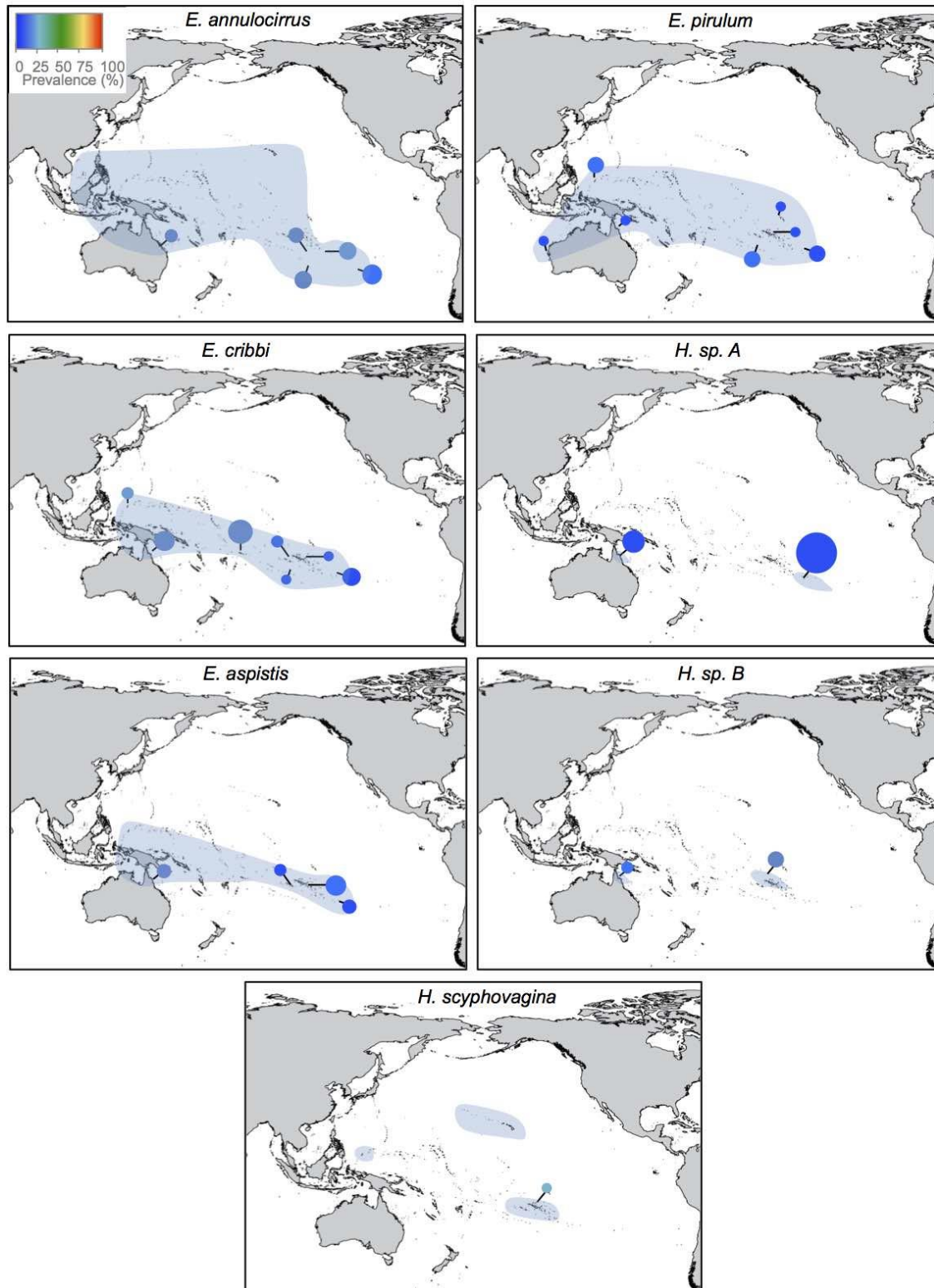
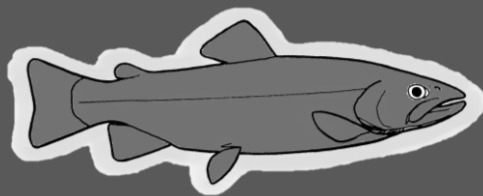


Figure S1. Areas of distribution (blue shadow), prevalence (circle colour) and abundance (circle size) of rare dactylogyrid parasites (< 20% prevalence) from butterflyfishes in the Indo-West Pacific Region.



CHAPTER III

External (skin and gills) fish mucus; biological and ecological importances, what is known and what is next?

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External (skin and gills) fish mucus; biological and ecological importances, what is known and what is next?

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Abstract

Fish mucosal surfaces have long been studied for their rheological properties and their major physiological roles in osmotic regulation and nutrient and gas exchange. However, numerous studies confirm that fish mucus is a complex biological material with relevant biological and ecological importance. Mucosal surfaces are mainly composed of glycoproteins, but also contain numerous other molecules like immune-related proteins and antimicrobial peptides (AMPs), which protect fish against infections; toxins, which mainly act as predator repellent and mycosporine-like amino acids (MAAs) that protect fish against UV radiation. Research on bioactive fish mucus components (mainly AMPs) is rapidly expanding due to their potential use in aquaculture and human medicine. However, the study of other mucus components and their involvement in intra and interspecific communication between organisms remains poorly understood. Finally, fish mucus naturally harbors a great quantity of microorganisms, whose roles are still unknown but most probably contribute to mucus balance and biological activities. This article explores external (skin and gills) fish mucus composition and discusses for the first time the associated biological and ecological roles both within fish and as a tool for chemical mediation between fish and environment. It also identifies novel priorities on fish mucus research, such as the use of the “omics” sciences for mucus molecules discovery and microbiome study.

Keywords: chemical mediation, fish mucus, interspecific communication, mucus microbiome, mucus molecules, mucus bioactivities.

1. Introduction

To better understand ecosystem dynamics, it is necessary to understand the complex network of interactions between organisms (Rossberg 2012). Fish are among the most biologically complex organisms inhabiting marine ecosystems, presenting specialised organs and a central nervous system like higher vertebrates with well-developed sensory systems (Bone and Moore 2007). Fish biology and ecology have been studied for a long time, and there is extensive information of intraspecific communication between fish (Timms 1975). Studies have shown that fish interact through visual, acoustic, electric or chemical signals (e.g., Sorensen and Stacey 2004; Moller 2006). However, there is still little knowledge on how interspecific interactions occur and what are the signals involved. Some studies have shown that interspecific communication mainly occurs through chemical signaling (Wisenden 2007; Hay 2009). For example, Kidawa et al. (2008) showed that blood from Black rockcod (*Notothenia corriceps*, Nototheniidae) acted as a chemical attracting signal to several necrophagous invertebrate species. However, there are very little studies where the chemical molecules involved in interspecific communication in fish have been identified and characterised, most probably because of the biological complexity of fish, rendering chemical isolation especially difficult (Ito et al. 2006; Ohashi et al. 2007).

In fish, the mucosal layers recovering the epidermis and gills is the main surface of exchange between them and their surrounding environment. Several studies have shown fish mucus involvement in intra and interspecific chemical communication (Todd et al. 1967; Bekliougu et al. 2006). Mucus acts as a thin dynamic physical and biochemical barrier that enables nutrient, water, gas and molecule diffusion (Shepard 1993). Mucus displays numerous biological and ecological roles such as osmoregulation (Shephard 1993), protection against abrasion (Oosten 1957), protection against environmental toxins and heavy metal toxicity (Coello and Khan 1996), parental feeding (Chong et al. 2006), protection against pathogens (Gómez et al. 2013) and chemical mediation (Leonard et al. 2012).

Teleost mucus is very similar to mammalian mucus and is mainly composed of highly glycosylated glycoproteins called mucins (Shephard 1993). Fish mucus contains numerous immune molecules such as lysozymes, immunoglobulins, complement and lectins, antimicrobial peptides (AMPs) and antimicrobial proteins (Esteban 2012). Other molecules present in fish mucus include mycosporine-like amino acids (MAAs), toxins or kairomones

(uncharacterized molecules that serve as infochemicals to other species) (Shephard 1993; Purcell and Anderson 1995).

Interest in fish mucus has been growing in the last decade due to the discovery of numerous bioactive molecules (antibacterial, antiviral, antifungal and antiparasitic) and their potential applications to human medicine and aquaculture (Rakers et al. 2013; Beck and Peatman 2015). Most studies have focused on immune-related molecules and AMPs, however, few studies have analysed other mucus molecules and their ecological roles in the environment. Secondary metabolites, for example, even though they are known to play key roles in the communication of a wide range of species including plants, invertebrates and microorganisms have been rarely studied in fish tissues or mucus (Hay et al. 2009).

There are numerous research and review articles studying specific topics on fish mucus (e.g. Rakers et al. 2013; Lazado et al. 2014; Larsen et al. 2015). However, none of them have studied fish mucus under a holistic approach, assessing its importance both for the fish and the ecosystem. This original critical synthesis aims to gather and analyze most of the current knowledge on skin and gill fish mucus, in order to highlight fish mucus importance in the marine ecosystem as well as identify the gaps in knowledge and provide future directions. We discuss the mucus composition, including the hardly studied mucus microbiome and mycobiome, and we discuss for the first time the fish mucus biological and ecological roles in species interactions. We also examine the state of fish mucus research and identify novel research priorities, focusing on the use of “omics” technologies to discover novel mucus molecules and study the mucus microbiome.

2. Mucus composition and biological activities

Mucus is produced by goblet and club cells found in the epithelium and it is constantly being secreted and shed, rendering pathogen infections difficult (Shephard 1993; Cone 2009). Mucus composition and its rheological properties are vital for the maintenance of mucus functions (Lai et al. 2009). For example, mucus viscoelasticity determines the ability to block many types of motile bacteria (Cone 2009), and decreased secretion rates have been associated with increased infection rates (Tabak 1995).

Numerous biological activities have been found in fish mucus, contributing greatly to recent development of fish mucus research and rendering fish mucus molecules (e.g. AMPs) interesting to veterinary and human medicine research. Antibacterial, antifungal and antiviral activities against human and fish pathogens have been found in many species of fish as well as proteolytic, cytotoxic, pro-apoptotic and antitumor activities (e.g. Hellio et al. 2002; Kwak et al. 2015). Even if mucus composition has been studied intensively, especially its antimicrobial compounds, there is still limited knowledge on most of the fish mucus components. Here, we summarise the different identified fish mucus components to date, and discuss their biological roles and the gaps in knowledge (Figure 1 – mucus composition and ecological roles).

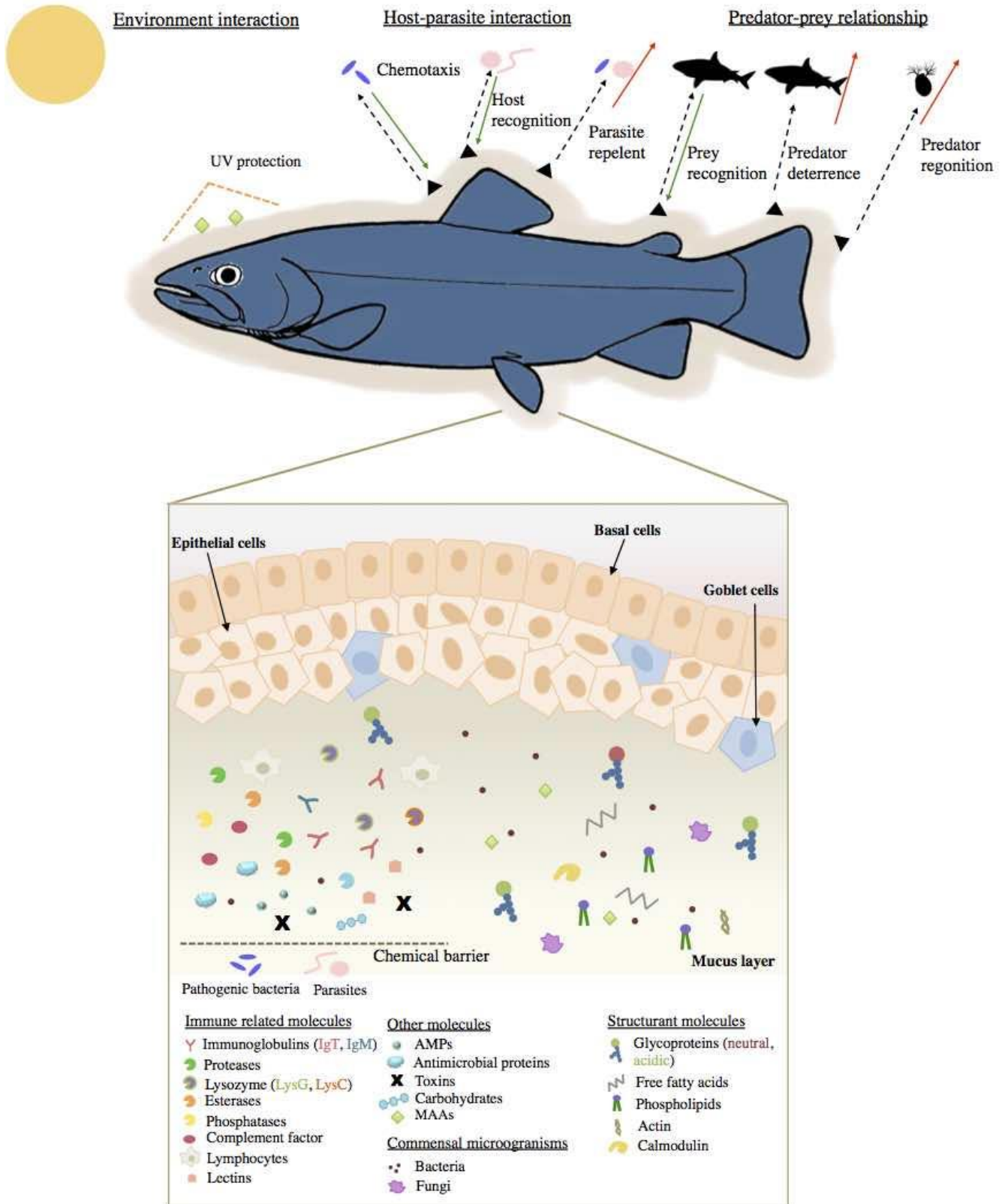


Figure 1. Chemical and biological compositions of epidermal fish mucus and its ecological interactions.

2.1 Glycoproteins and other carbohydrate derivatives

Fish mucus is mainly constituted by high molecular weight, filamentous, O-glycosylated proteins (GPs) also called mucins. GPs are responsible for the viscoelastic and adhesive properties of mucus. Since glycoprotein characteristics depend on their carbohydrate chains, characterisation of the carbohydrate chains is important to better understand mechanisms of fish disease resistance (Estensoro et al. 2012; Pérez-Sánchez et al. 2013).

Several studies showed differences in glycoprotein composition depending on the species habits and habitat characteristics (Gona 1979), but also between different fish tissues (epithelium, gills and intestines) (Jin et al. 2015). For example, Kumari et al. (2009) found differences in the composition and concentration of GPs between the epithelium of the gill arches and the gill rakers (abundance of GPs with O-sulphate esters) and the gill fillaments and the secondary lamellae (abundance of GPs with sialic acid) in the catfish *Rita rita* (Bagridae), indicating an adaptation of mucus viscosity to optimise physiological functions.

Based on their structural and functional features, mucins are grouped in two different families: secreted mucin glycoproteins, which are highly O-glycosylated proteins assembled in oligomers giving mucus its viscosity (Thornton et al. 2008), and membrane-bound forms, which are cell-surface mucins containing an extracellular O-glycosylated domain and can form long filaments carrying oligosaccharides, which can act as ligands in microbial adhesions (McGuckin et al. 2011; Beck and Peatman 2015). Histochemical studies have shown presence of neutral and acidic mucins, the latter including both sialomucins and sulfomucins (Mittal et al. 1994).

Ebran et al. (2000) and Molle et al. (2008) isolated and characterised novel glycoproteins from different fish skin mucus with strong pore-forming and antibacterial activities. Transferrin, a globular glycoprotein with a high iron chelator power, was also identified in the mucus of Atlantic cod (*Gadus morhua*, Gadidae) and Atlantic salmon (*Salmo salar*, Salmonidae), suggesting an involvement in innate immunity (Ræder et al. 2007; Easy et al. 2012). However, even if there is extensive literature on the subject, the chemical composition and properties of most GPs in fish mucus remain poorly understood (Sumi et al. 2004).

Besides GPs, other types of carbohydrate molecules exist in fish mucus. Qin et al. (2002a) isolated a novel polysaccharide from Pond loach (*Misgurnus anguillicaudatus*, Cobitidae), known as misgurnan or MAP. MAP proved to have multiple bioactivities, such as, antioxidant, apoptotic and immunomodulating activities (Qin et al. 2002b; Zhang and Huang 2005a, 2005b).

2.2 Proteins and peptides

Mucosal tissues in fish contain a very diverse array of proteins, including some structural proteins, immune-related proteins and antimicrobial peptides, which display varied biological roles within the fish biology.

Although, reports of structural proteins in mucus are scarce, some studies have shown the presence of high levels of actin in fish epidermal mucus, suggesting that actin may have a discrete role in mucus structure (Easy and Ross 2010). Keratin and calmodulin were also identified in salmon epidermal mucus (Easy and Ross 2010). Presence of calmodulin in fish mucus suggests an enzymatic involvement in the control of integumental permeability (Flik et al. 1984). Cholinesterases have also been identified in skin mucus of carps, and it has been suggested that they can interact with organophosphate pesticide molecules and thereby neutralise them and decrease skin penetration (Nigam et al. 2014).

Recently, the use of new techniques such as proteomics has enabled the identification of many proteins unreported to date in fish mucus, including some immune-related proteins (galectins, caspases, concanavalin A, FK-506 binding protein, cyclophilin A and cystitis B), proteins involved in cellular metabolism (ubiquitin, glutathione hydrolase and transferase and calreticulin), carbohydrate metabolism (citrate synthase and enolase and glyceraldehyde-3-phosphate dehydrogenase) and proteins involved in lipid metabolism (preapolipoprotein Aa and fatty acid-binding protein) (Rajan et al. 2011, 2013; Easy et al. 2012, Cordero et al. 2015).

2.2.1 Immune related proteins

Fish mucus is enriched with a multitude of immune related proteins such as lysozymes,

phosphatases, esterases, proteolytic enzymes, complement factors, lectins, immunoglobulins, C-reactive proteins and lymphocytes that attempt to eliminate pathogens and launch the immune cascade when an infection occurs (Guardiola et al. 2014).

Lysozyme, is a well-studied bacteriolytic enzyme that acts disrupting mucopolysaccharides in the bacterial cell walls and thus, cause bacteria death (Saurabh and Sahoo 2008). Two isoforms of lysozyme that are similar to goose (g) type and chicken (c) type in vertebrates have been detected in fish mucus (Nigam et al. 2012).

Acid and alkaline phosphatases and esterases are also important enzymes found in fish mucus; however their function in fish mucosal immunity is not completely understood (Nigam et al. 2012). Alkaline phosphatases in mucus have shown to act as antibacterial agent, to play a protective role in the initial stages of wound healing and to be potential stress indicators in skin mucus of fish (Rai and Mittal 1983; Ross et al. 2000).

Proteases provide protection against fish infections, either directly, by cleaving pathogen proteins, or indirectly, by modifying mucus consistency and activating the production of other immune molecules such as complement, immunoglobulins or AMPs (Esteban 2012). Different types of proteases (trypsins, metalloproteases, cathepsins and aminopeptidases) have been identified in fish skin mucus (Esteban 2012). Serine- and metalloproteases have been reported as the predominant proteases in several fish species (Salles et al. 2007; Tvette and Haugan 2008). Metalloproteases are associated with the immune response, tissue repair and in the activation of pro-cathepsin D in cathepsin D, a protease that regulates secretion of AMPs (Cho et al. 2002; Pedersen et al. 2015).

The complement system plays an important role in the innate immune system and elimination of pathogens in fish (Boshra et al. 2006). And some studies have identified complement components present in fish mucus, such as C7 in skin and intestine mucus of grass carp, C3 in skin mucus of Atlantic halibut (*Hippoglossus hippoglossus*, Pleuronectidae) and C1q in skin mucus of Siberian sturgeon (*Acipenser baerii*, Acipenseridae) (Shen et al. 2012; Fan et al. 2015).

Fish mucus also contains a wide variety of lectins (e.g. Suzuki et al. 2003; Tsutsui et al. 2011). Several studies have shown that lectins in fish mucus are able to agglutinate several bacterial strains or bind to other parasites such as the parasitic nematode *Heterobothrium*

okamotoi, suggesting the important role of mucus lectins in host defences (Tsutsui et al. 2006).

Natural antibodies such as immunoglobulins are also present in fish mucus and they play a key role both in innate and adaptive immunity, producing specific antibody responses against various antigens (Coscia et al. 2014). Although three types of immunoglobulins have been identified in teleost fish, (IgM, IgT and IgD), only IgM and IgT have been found in fish mucus (Sunyer 2013). IgT seems to play a major role in fish mucosal immunity, while IgM is present at low levels in mucosal surfaces (Zhang et al. 2010; Xu et al. 2013).

Although most immune-related proteins in fish mucus have been discovered by classic biochemical approaches, the use of new techniques such as genomics, transcriptomics and proteomics is allowing researchers a more rapid and efficient identification of fish mucus immune-related molecules, as well as elucidating their sources of variability and biological roles (Cordero et al. 2015).

2.2.2. AMPs and antimicrobial proteins

An important component of fish mucus with multiple biological activities are antimicrobial peptides (AMPs). AMPs are nearly found in all life forms and are normally small cationic peptides (less than 50 aminoacids, < 10 kDa), amphipathic and encoded by single genes (Zaslhoff, 2002). AMPs typically exhibit activities against multiple pathogens including Gram-positive and Gram-negative bacteria, fungi, viruses and parasites. Certain AMPs can also function as immunomodulators (Bowdish et al. 2005). Interest in fish AMPs, and especially in fish mucus AMPs has risen recently, for their potential use both in aquaculture and human medicine as antimicrobial agents, vaccine adjuvants, inactivated vaccines, antitumor agents and even in dermatology (Noga et al. 2011; Rajanabu and Chen 2011; Rakers et al. 2013).

Although AMPs are highly heterogeneous in their primary structure, three main classes of AMPs have been described in teleost fish (α -helical peptides, cysteine-rich AMPs and AMPs derived from functional proteins). α -helical peptides, comprise the most common AMP family in fish, the piscidins, peptides with a short, linear structure, which adopt α -helical conformation when interacting with bacterial membranes (Smith et al. 2010). The piscidins family includes the moronecidins (piscidins), pleurocidins, dicentracins and chrysopsins

(Masso-Silva and Diamond, 2014). Several immunohistochemistry and genomic studies have reported piscidins to be widespread among teleost fish (Silphaduang et al. 2006; Salerno et al. 2007). Piscidins 1 and 2 (Pis1 and Pis2 genes) have been found in Atlantic cod epidermal mucus and whereas Pis2 was found expressed similarly in all tissues, the strong expression of Pis1 in mucus suggests its involvement with systemic and mucosal immunity (Ruangsri et al. 2012). Piscidins present broad antibacterial, antifungal, antiparasitic and antitumoral activities (Masso-Silva and Diamond, 2014). Pleurocidins, first isolated from the skin mucus of winter flounder (*Pleuronectes americanus*, Pleuronectidae), have broad-spectrum antimicrobial activities and inhibit DNA, RNA and protein synthesis (Cole et al. 1997, 2000). Dicentracin is a peptide isolated from several organs of European sea bass (*Dicentrarchus labrax*, Moronidae), including skin and gills and seems to have an important defensive role in the fish mucosal tissues (Salerno et al. 2007). Chrysopsin is also an AMP from the piscidin family found in mucus of European sea bass (Fekih-Zaghib, 2013). Other linear AMPs described in fish mucus include pardaxin from the skin mucus of the Moses sole (*Pardachirus marmoratus*, Soleidae) and pelteobagrins from the skin mucus of the yellow catfish (*Pelteobagrus fulvidraco*, Bagridae) (Oren and Shai, 1996; Su, 2011).

Amongst the cysteine-rich AMPs in teleost fish are three families: defensins, cathelicidins and hepcidins (Smith et al. 2010). The most prominent group of cysteine-rich AMPs are the defensins. Several β -defensin genes have been identified in mucosal tissues of several fish species and besides their broad antibacterial and antiviral activity they seem to be an important component of the innate defences of fish (Casadei et al. 2009; Marel et al. 2012, Ruangsri et al. 2013). Although some cathelicidins have been reported in several fish species, little is known about their function and importance in fish (Scocchi et al. 2009). To date, cathelicidins have not been reported in fish mucus, however, Chang et al. (2005) found expression of a cathelicidin gene (rtCATH_1) on the skin of the rainbow trout. Hepcidins are highly disulphide bonded β -sheet peptides, and have been identified in various tissues of fish, including skin; although, higher expression levels have been found in liver and head kidneys (Huang et al. 2007; Yang et al. 2007). AJN-10 is a novel cysteine-rich AMP isolated from the skin mucus of Japanese eel (*Anguilla japonica*, Anguillidae) (Liang et al. 2011).

While most AMPs are derived from a biologically inactive proprotein that is processed to the active form, some AMPs are derived from larger, functional proteins that have primary functions other than immune defense (Ullal et al. 2008). The most common of these are

histone-like proteins. Histones are mainly known for their involvement in nuclear regulation and as an integral part of chromatin structure, however, more recent studies have shown they present strong antibacterial activities suggesting they may play a role in innate defence in many animals (Parseghian and Luhrs 2006; Noga et al. 2011). Several histones (H1, H2A, H2B) with antibacterial, antifungal and antiparasitic activities have been identified in skin mucus of several fish families including Salmonidae, Gadidae and Ictaluridae (Robinette et al. 1998; Fernandes et al. 2002; Bergsson et al. 2005). Also, an increasing number of AMPs derived from terminal parts of histones (N-terminal and C-terminal regions) and other proteins have been described including parasin-I from the skin mucus of catfish (*Parasilurus asotus*, Siluridae), hipposin from the Atlantic halibut, SAMP H1 from Atlantic salmon and onchorrycin II from the rainbow trout. (Park et al. 1998; Birkemo et al. 2003; Fernandes et al. 2004; Lüders et al. 2005).

Finally, other proteins displaying antibacterial properties in the skin mucus of several fish species include L-amino acid oxidases (LAOs), like *Sebastes schlegeli* antibacterial protein (SSAP) from rockfish (*Sebastes schlegeli*, Sebastidae), ribosomal proteins like L40, L36A, L35 and S30 from Atlantic cod and rainbow trout and hemoglobin-like proteins (Hb- β) from channel catfish (Fernandes and Smith 2002; Bergsson et al. 2005; Kitani et al. 2007; Ullal et al. 2008).

Although, research on fish mucus AMPs is rapidly progressing, most studies are essentially descriptive and focused on characterisation of new AMPs in single fish species. Despite the vast diversity in fish AMPs, studies show that AMPs are evolutionary well conserved, and many fish AMPs are preserved in higher vertebrates (Rakers et al. 2013). However, besides piscidins, most fish AMPs have been described in very few species, and there is still little knowledge on their distribution among fish families. In the future, fish mucus AMP research should focus on better understanding AMP evolution in fish, and their spread among fish families. Fish mucus AMPs show great potential as antimicrobial agents for therapeutic applications, however, to become exploitable, more research has to be conducted to understand their origin and mode of action in order to identify the conserved or most active parts.

2.3 Lipids

Lipids in fish mucus have been very rarely studied, however those studies indicate that they are also major constituents of fish mucus. Lewis (1970) showed that lipid quantity in fish mucus is surprisingly high when compared to human sebum, being phospholipids (lecithin and phosphatidyl ethanolamine) the most abundant lipids. Other lipids present are squalene, sterols, triglycerides, free fatty acids and carotenoids like β -carotene (Lewis 1970; Jais et al. 1998). It seems that free fatty acids might have protective functions and the interaction between the phospholipids and glycoproteins may determine the relative viscosity of mucus (Lewis 1970).

2.4 Toxins and other specific molecules

To date, fish mucus research has focused mainly on the study of macromolecules. Nevertheless, fish mucus is a rich and yet unexplored source of novel bioactive molecules. For example, fish mucus toxins or mycosporine like amino acids (MAAs) have been described in fish mucus before, but they have rarely been integrated in studies of fish mucus composition and roles (Table 1).

Fish toxins are bioactive secretions normally stored in specialized glands but frequently found in fish epidermal mucus, which possess a variety of biological functions such as defence and competitor and predator deterrence (Baumann et al. 2014). Ichtyocrinotoxins (fish epidermal toxins not associated with any venom apparatus) with very diverse structures have been reported in over 50 species of teleost fish from 14 families (Halstead, 1970; Munday et al. 2003) (Table 1). Most crinotoxins are released into mucus layers when fish are stressed or injured, and they normally have hemolytic activity and can have antibacterial properties (Nair, 1988; Sugiyama et al. 2005).

Tetrodotoxin (TTX), secreted by numerous pufferfish species (Tetrodontidae and Diodontidae), is one of the best studied marine fish crinotoxins (Williams, 2010; Figure 2). TTX is a neurotoxin that inhibits nerve and muscle conduction by selectively blocking sodium channels, resulting in respiratory paralysis that causes death (Narahashi, 2008). TTX has been identified in multiple organisms such as starfish and mollusks, but also in terrestrial invertebrates and vertebrates (Noguchi and Arakawa, 2008; Stokes, 2013). Studies have

suggested bacteria as the primary source of TTX, and have found that TTX accumulation in pufferfish occurs via the food chain (Noguchi and Arakawa, 2008). Although, the origin of most ichthyocrinotoxins remains unstudied, it is likely that like TTX, most fish mucus toxins are either produced by symbiotic microorganisms or accumulated by other means (Daly et al. 2004).

Fish family	Fish species	Toxin	Molecule type	Reference
Tetraodontidae	<i>Takifugu niphobles</i> , <i>T. pardalis</i> , <i>T. vermiculare porphyreum</i> , <i>T. poecilonotus</i> , <i>T. vermiculare radiatum</i> , <i>T. xanthopteron</i>	Tetrodotoxin	Amino perhydroquinazoline	Kodama et al. 1986; Ito et al. 2006
	<i>Teatrodon</i> sp.	Saxitoxin, decarbamolysaxitoxin	Purine base with 3 carbon bridge linking positions 3 and 9 and a methyl carbamate at position 6	Nguyen et al. 2008
Ostraciidae	<i>Ostragon lentiginosus</i> , <i>Lactophrys triqueter</i>	Ostracitoxin (also called Pahutoxin)	Choline chloride ester of a fatty acid	Thompson, 1964; Boylan and Scheuer, 1967
	<i>O. immaculatus</i> , <i>O. lentiginosus</i>	Pahutoxin and homopahutoxin	Fatty acid derivative	Fusetani and Hashimoto, 1987
	<i>O. cubicus</i>	Boxin	Protein	Kalmanzon and Zlotkin, 2000; 2001
Aracaniidae, Ostraciidae	<i>Anoplacapros lenticularis</i> , <i>Aracana aurita</i> , <i>Aracana ornata</i> , <i>Lactoria fornasini</i> , <i>O. cubicus</i> , <i>Rhinesomas reipublicae</i> , <i>Strophuichthys inermis</i> , <i>S. robustus</i>		β -substituted choline chloride esters of palmitic acid	Goldberg et al. 1988
Soleidae	<i>Pardachirus marmoratus</i>	Pardaxins (I-II)	Polypeptide (AMP)	Lazarovici et al. 1986
	<i>P. pavonicus</i>	Pavoninins 1-6	Steroid saponins	Tachibana, 1984; Williams and Gong, 2007
Plotosidae	<i>Plotosus lineatus</i>	Toxin I, II	Natterine-like (proteinaceous)	Tamura et al. 2011
Ariidae	<i>Cathorops spixii</i>	WAP65	Protein	Ramos et al. 2012
Potamotrygonidae	<i>Potamotrygon</i> gr. <i>orbigny</i>	Orpotrin, porflan	Peptides	Conceição et al. 2006
Serranidae	<i>Grammistes sexlineatus</i> , <i>Pogonoperca punctata</i>	Grammistsins (A-E, 1, 2, Pp1, Pp2, Pp3, Pp4a, Pp4b)	Polypeptides	Hashimoto and Oshima, 1972; Shiomi et al. 2000, Sugiyama et al. 2005

Gobiesocidae	<i>Diademichthys lineatus</i>	Type of grammistin	Polypeptides	Hori et al. 1979
Gobiidae	<i>Gobiodon spp.</i>	Type of grammistin	Polypeptides	Hashimoto et al. 1974

Table 1. Toxins described in fish mucus.

Recent studies have shown that fish mucus contains UV absorbing molecules, which have been identified as micosporine-like amino acids (MAAs), which are water-soluble nitrogenous compounds that perform a photo protective function against solar radiation (Zamzow, 2007). MAAs are selectively sequestered and accumulated via trophic transfer from corals and producer species (Mason et al. 1998). Three different MAAs have been identified to date in fish mucus: palythene, asterina-33 and mycosporine-N-methylamine serine. Different fish species present different combinations of MAAs (Eckes et al. 2008; Figure 2). Finally, fish mucus it is highly likely to contain other specialised and yet unknown metabolites, that could be 1) produced by the fish itself, 2) produced by associated microbiota or 3) accumulated in the mucus by other means such as diet.

2.5 Mucus microbiome

Living organisms harbor a diverse community of microorganisms that have important functions in maintaining the hosts' health and homeostasis (Maranduba et al. 2015). In fish, native microbiota influence the innate immune system and thus is involved in disease resistance (Gomez and Balcazar, 2008). Although interest in organisms' microbiota and microbiome has exploded in the last decade, studies exploring fish mucosal microbiota remain limited (Llewellyn et al. 2014; Table 2).

Several studies have shown that the composition of the fish mucosal microbiota is highly variable. Higher bacterial diversity has been found in external organs such as skin or gills than the gut (Smith et al. 2007; Lowrey et al. 2015). Other studies showed that bacterial population associated with epidermal fish mucus was species specific and did not reflect the bacterial communities in the surrounding water (Smith et al. 2007; Larsen et al. 2013). Besides, some studies point out mucus microbiome as highly dynamic, dominant bacterial taxa changing upon environmental conditions such as seasonality, suggesting the most adapted bacteria to a

certain environment would appropriate the most niche space (Wilson et al. 2008; Larsen et al. 2015; Schmidt et al. 2015)

Commensal microbiota in mucosal surfaces are suggested to provide protection against opportunistic infection. Some studies showed that when microbiota homeostasis was disrupted (dysbiosis) by stressful conditions, the abundance of probiotic-like bacteria decreased while pathogenic bacteria increased (Boutin et al. 2013). Boutin et al. (2014) showed a strong negative correlation between the indigenous bacteria *Methylobacterium* and the pathogenic *Flavobacterium* suggesting a mutually competitive relationship. *Methylobacterium rhodesianum* is known to produce a poly- β -hydroxybutyrate, a polymer of short-chain fatty acid that inhibits growth of bacterial pathogens like enterobacteria and *Vibrio* sp. (Halet et al. 2007).

To date, very few studies have analysed the potential of vertebrate-associated microorganisms to produce bioactive compounds. Recent studies are revealing that microorganisms are the main producers of most of the natural products found in marine invertebrates (Balskus 2014). Since vertebrate organisms such as fish possess diverse microbiomes, it is likely to think that microorganisms could also synthesize bioactive secondary metabolites. Sanchez et al. (2012) showed that some isolated bacterial strains from fish intestines possessed antibacterial activity and they identified a novel bioactive lipid, sebastenoic acid, as antimicrobial agent (Figure 2). Another study analysed the rainbow trout microbiome and found antifungal activity in two *Arthrobacter* sp. skin isolates, a *Psychrobacter* sp. strain and a combined skin aerobic bacterial sample (Lowrey et al. 2015).

Most studies on the associated microorganisms from animals have focused on bacteria, however, very recent studies are showing the key role of commensal fungi (mycobiota) and their effect on host immune system and health (Underhill and Iliev 2014). Most of those studies have focused on human organs and human mucosal surfaces, but some studies are starting to investigate the mycobiome of marine species (Borzykh and Zvereva 2015). Moreover, symbiotic fungi might also be a potential source for bioactive natural products that might benefit their hosts (Debbab et al. 2012). Therefore, it will be of great importance to study the fish mucus mycobiota to gain a better understanding of the whole mucosal system, its interactions with other microorganisms (commensal and pathogenic) as well as their involvement in the fish immunity and health.

Fish species	Predominant bacteria phyla (genus)	Tissue	Reference
<i>Lutjanus campechanus</i>	Proteobacteria (Vibrio, Photobacterium)	Skin mucus	Arias et al .2013
<i>Merlangius merlangus</i>	y- Proteobacteria (Photobacterium)	Skin mucus	Smith et al. 2007
<i>Mugil cephalus</i>	Proteobacteria	Skin mucus	Larsen et al.2013
<i>Fundulus grandis</i>	y-proteobacteria (Pseudomonas), cyanobacteria (Prochlorococcus)	Skin mucus	Larsen et al. 2015
<i>Megalobrama amblycephala</i>	y-Proteobacteria (Acinetobacter) and firmicutes (Anoxybacillus flavithermus)	Gills	Wang et al. 2010c
<i>Megalobrama amblycephala</i>	y-Proteobacteria (Acinetobacter)	Skin	Wang et al. 2010c
<i>Carassius auratus</i>	y-Proteobacteria (Acinetobacter) and firmicutes (Anoxybacillus flavithermus)	Gills	Wang et al. 2010c
<i>Carassius auratus</i>	Proteobacteria (Enterobacter) and firmicutes (Staphylococcus)	Skin	Wang et al. 2010
<i>Salvelinus fontinalis</i>	α -proteobacteria	Skin mucus	Boutin et al. 2013; 2014
<i>Salmo salar</i>	α -proteobacteria (Pseudomonas)	Skin mucus	Cipriano, 2011
<i>Gadus morhua</i>	y- proteobacteria (Photobacterium)	Skin mucus	Wilson et al. 2008

Table 2. Bacteria found in external fish mucus.

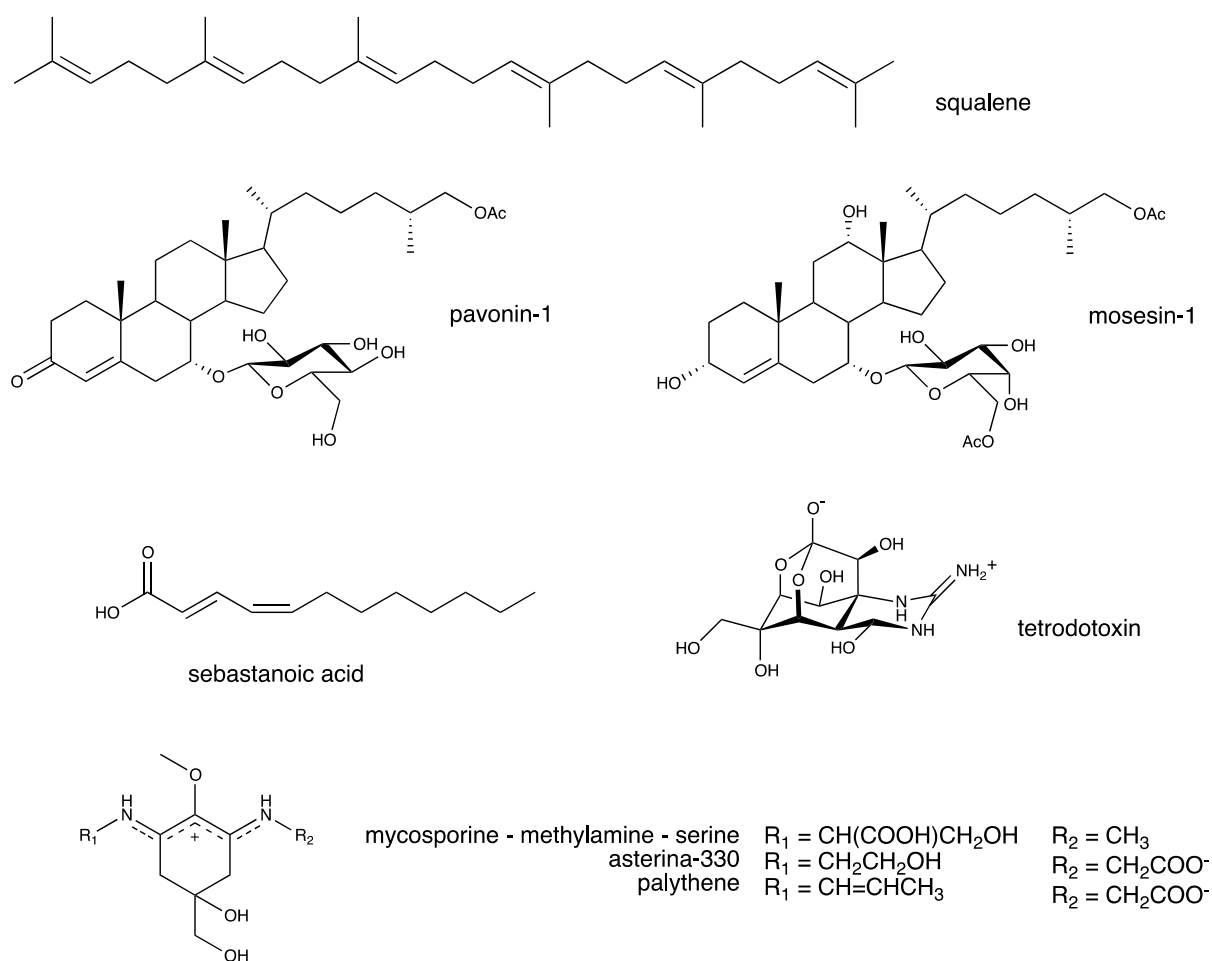


Figure 2. Small molecules found in epidermal fish mucus.

3. Mucus roles in ecological interactions

Molecules found in skin and gill fish mucus display key roles in the fish physiology and especially in fish protection against infections. However, some of those molecules also possess very important ecological roles in the ecosystem as a tool for communication. Studies have shown that epidermal and gill mucus substances can act as infochemicals in different interspecific interactions, such as in prey-predator relationship or parasite-host interaction (Figure 1). Fish mucus molecules can be perceived by a wide range of organisms and generate different type of responses. Several studies have shown that different pathogenic bacteria exhibit positive chemotaxis towards their hosts' mucus (Bordas et al. 1998; Larsen et al.

2001). Although specific molecules responsible for this activity have not yet been identified, O'toole et al. (1999) suggested free amino acids and carbohydrates could act as chemoattractant molecules in fish mucus while Klesius et al. (2008) suggested that a lectin-like substance may be responsible for the chemotaxis.

Chemodetection of fish mucus and behavior modification has also been reported in other parasites such as the actinospores from oligochaetes, myxozoans and ectoparasites like copepods and monogeneans (Xiao and Dresser 2000; Ohashi et al. 2007; Kallert et al. 2011; Brooker et al. 2013). Kallert et al. (2011) found that free nucleosides present in trout mucus (inosine, 2'-deoxyinosine and guanosine) stimulated myxozoan attachment. Another study showed the glycoprotein WAP 65-2 in tiger puffer fish (*Takifugu rubripes*, Tetradontidae) skin mucus induced attachment of *Neobenedeniagirellae* oncoramicidia (Monogenea, capsalidae) (Ohashi et al. 2007). It has also been proposed that TTX from pufferfish may also act as an attractant of the infective copepodid stages of *Pseudocaligus fugu* (Ito et al. 2006).

Fish mucus substances act often as semiochemicals in predator-prey relationships, either as predator deterrents or predator and prey signaling. For example it is known that pavoninins, pardaxin and mosesins secreted by several species of soles repel sharks, by acting on their olfactory sense (Tachibana et al. 1984) (Table 1, Figure 2). TTX from puffer fish has also been reported to repel predation by groupers and act as a sex pheromone attracting males towards fertile females (Matsumura 1995; Williams 2010). Itoi et al. (2014) suggested that the female parent transferred vertically TTX to the larvae (through TTX accumulation in the ovaries) to increase egg and larvae survival.

Purcell and Anderson (1995) found that *Physalia physalis* could identify its preys by chemical cues from fish epidermal mucus, although the chemical nature of these molecules remains unknown. Several organisms are also able to identify fish mucus chemicals to detect predators and avoid predation. Several studies indicate that nocturnal diel vertical migration (DVM) in zooplankton is affected by uncharacterised kairomones present in mucus of planktivorous fish (Forward and Rittschof 2000; Beklioglu et al. 2006). Forward and Rittschof (2000) proposed that disaccharide degradation products of predator mucus containing sulfated and acetylated amines can serve as kairomones, whereas Beklioglu et al. (2006) suggested that both fish and mucus-dwelling bacteria interact in the release of kairomones. Recent research showed that the polychaete *Nereis* developed chemosensory

mechanisms for predator detection (chemical cues from fish mucus) to minimise predation risks (Schaum et al. 2013).

Goby epidermal toxins and their ecological implications are well studied and provide a good example of the importance and complexity of fish mucus molecules in ecosystems dynamics. Coral gobies are known to possess different epidermal mucus toxins that exhibit predator deterrence and parasite avoidance (Munday et al. 2003; Gratzner et al. 2015). Recent studies have shown how goby mucus substances play a key role in the mutualistic association between gobies and corals. Dixon and Hay (2012) showed that *Acropora*, chemically attracted gobies when attacked by toxic seaweeds. In turn, gobies would trim the seaweed and increase their own mucus toxicity, protecting them against predators and parasites. Dirnwoeber and Herler (2013) also showed that goby skin mucus functioned as a corallivore repellent, protecting corals from predation. Although, role of goby epidermal mucus toxins has been ecologically well studied, their chemical structures are still unknown.

The study of chemical mediation in marine ecosystems is vital to understand ecosystem dynamics and thus understand how ecosystems can respond and adapt to changing conditions. Fish chemical mediation is still poorly understood, with very few examples where fish mucus molecules have been elucidated and their ecological roles identified. As previously shown for TTX, a single molecule can have multifunctional properties. Species, sex, and tissue specific differences in the distribution of TTX render unclear the exact function of this molecule in pufferfish. Depending on the concentration, TTX may function as a chemical defense against predators, as attractant for parasites or as a chemical pheromone during spawning. These molecules of keystone significance are vital in structuring ecological communities, and therefore a deeper understanding is needed. (Ferrer and Zimmer 2013). Research on fish mucus, coupled to the development of new technologies will allow to clarify how fish interact and respond to their environment.

4. Mucus dynamics

Mucus is a dynamic matrix and its composition varies among fish species, being influenced by endogenous factors such as sex and developmental stage and exogenous factors such as stress, water temperature, pH and infections (Esteban 2012).

Chong et al. (2005) found that adult fish presented higher amounts of protein in epidermal mucus than juvenile fish. Higher salinities have been associated with a decrease in density of mucus cells and an increase in mucus viscosity (Shephard 1993; Roberts and Powell 2005). Changes in protein levels and immune-related molecules have been observed upon different temperatures, with increased levels of lysozyme, hepcidin, IgM, lectin and mucosal antibodies at higher temperatures, which might be related to the increasing pathogen pressure in warmer temperatures (Huang et al. 2011; Jung et al. 2012; Ma et al. 2013). Toxic and irritating substances such as environmental contaminants produce irregular and excessive mucous secretions, which results in a decrease of mucus cell density after the chemical exposure, compromising fish health (Al-Zaidan et al. 2013).

Acute stress seems to cause changes in mucus proteins. Terova et al. (2011) showed an increase in the expression levels of histone-like proteins due to confinement, whereas, Easy and Ross (2010) observed changes in mucus proteases, in response to long-term handling stress in Atlantic salmon.

Several studies have shown that fish increase their mucus secretion as well as change their composition upon biological stressors such as pathogen presence and infection in order to better protect themselves (Gustafsson et al. 2013; Rajan et al. 2013). Van der Marel et al. (2010) showed that fish secreted higher quantities of mucins with higher glycosilation as an adaptation to higher bacterial loads in the water. Several studies have also showed a higher mucus production following parasites infection (Maki and Dickerson, 2003; Valdenegro-Vega et al. 2014). Higher quantities of immune-related molecules (lectins, lysozyme, immunoglobulins and cleaved transferrin) have been detected following different types of pathogenic infections (Easy and Ross 2010; Kar et al. 2015; Makesh et al. 2015). Finally, some mucus changes can be directly triggered by parasites, for example, monogenean and crustacean ectoparasites seem to modulate mucus production during attachment by reducing the density of fish mucus cells through disruption of cell dynamics within the epidermis in an attempt to minimise inhibitory effects of the mucus (Jones et al. 2001; Wells and Cone 2006).

Considering the mucus dynamics when studying fish mucus is important since they give insights on fish responses to environmental conditions. Modifications in fish mucus can compromise fish health but it could also affect or disrupt fish chemical mediations. Understanding mucus composition changes can be extremely useful in disease control

strategies in aquaculture, but also in conservation studies.

5. Research advances and priorities

Fish mucus research is rapidly growing (Makesh et al. 2015; Larsen et al. 2015). Technological advances in the “omics” sciences such as proteomics has recently allowed the discovery of many unreported proteins in fish mucus (Rajan et al. 2011). Developments in the field of genomics facilitate the study of genes present in fish mucus, and it is allowing the rapid expansion of microbiomics (microbiome characterisation). Studying fish mucus microbiome and mycobiome together with their roles and their associated secondary metabolites are some of the novel priorities in fish mucus research. The use of metabolomics could also be highly useful in fish mucus research. Firstly, on the discovery of small metabolites, which have been poorly studied, and secondly, metabolomics provide metabolic signatures at specific moments of time so it could cast light on mucus dynamics (Figure 3).

Finally, fish mucus research needs to be approached from an integrative perspective in order to study the whole mucosal system. Fish mucus is a very complex biological material, involved in numerous biological functions within the fish and the ecosystem, which needs to be more studied and understood in order to evaluate its full potential. Numerous fish mucus molecules have drawn attention as potential candidates for drug and vaccine development. Fish mucus plays an important role in intra and interspecific relationships, and therefore a better comprehension on those relationships could clarify methods of co-evolution between fish and pathogens as well as provide important information to understand species adaptation in changing environments (Figure 3). This article is the first attempt to study fish mucus under a holistic approach, combining current knowledge in different disciplines to critically discuss the biological and ecological importance of mucus, and identifying new opportunities in the research of fish mucus.

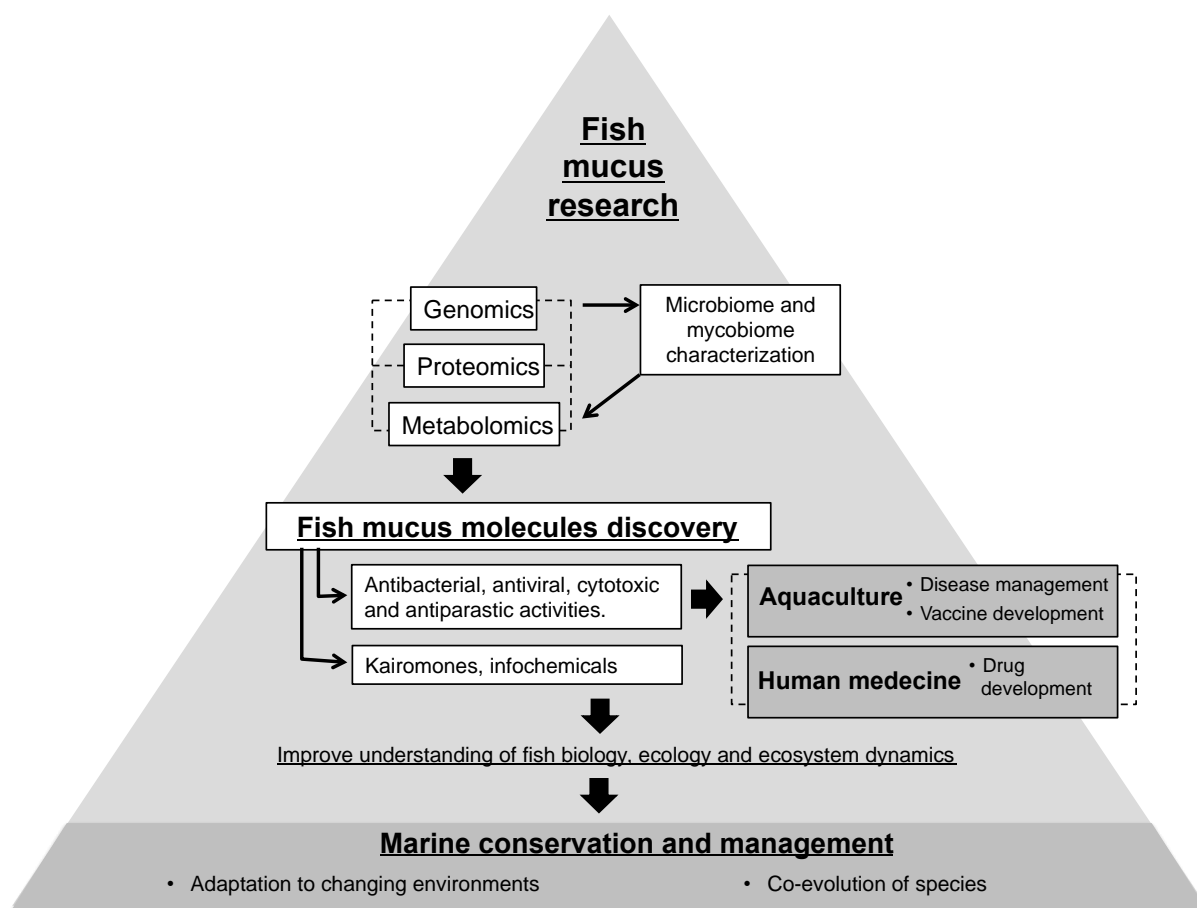


Figure 3. Diagram on the priorities of fish mucus research and applications.

6. Summary and conclusions

Fish mucus surfaces are dynamic layers that display very important functions in fish, playing major roles in physiological functions such as osmoregulation and protection against infections, but also as a tool of interspecific communication (Bekliougu et al. 2006). Mucus contains a wide variety of biologically active molecules that take part in numerous roles and biological interactions. Study of fish mucus components has exploded these last years for its potential use both in aquaculture and human medicine. However, as we stated in this article, there are numerous mucus components that need further research in order to better characterise the molecules present, but specially in order to elucidate their roles. We have discussed the multiple roles of mucus in organism interactions of several nature (predator-prey, parasite-host), highlighting mucus ecological importance and the need to identify the molecules responsible for these interactions. Elucidating the molecules responsible for the

chemical mediation between fish mucus and other organisms will allow a deeper understanding of marine ecosystem dynamics and thus will give new insights in both the aquaculture management of parasites but also in marine conservation studies. In addition, better understanding of mucus shifts and changes due to biological and physical stressors could help to prevent fish disease outbreaks but also monitor the health of the marine environment. Finally, nearly all studies have focused on the study of macromolecules, via multiple techniques such as proteomics, but little studies have tried to elucidate smaller specialised metabolites. We would like to draw attention to the importance of secondary metabolites and their varied bioactivities in better-studied species such as marine invertebrates and their potential presence and importance in fish mucus. Furthermore, the development of new disciplines such as metabolomics will allow studying fish secondary metabolites more efficiently. Recent studies have shown the importance of microbiome, and some bacterial synthesised molecules have been described (Sánchez et al. 2012). Therefore, the study of fish mucus microbiome and mycobiome is another promising field to both the discovery of new metabolites and better comprehension of the mucus entity and biological and ecological roles. To summarize, fish mucus is a key tissue in fish fitness and communication. Fish mucus contains a wide range of bioactive molecules that display numerous roles within the fish and other organisms and are of interest for medicine research. We propose here a new perspective on fish mucus research, using an integrative approach to elucidate new bioactive mucus molecules and microorganisms and study their biological and ecological roles using the “omics” technologies.



CHAPTER IV

Characterisation of the gill mucus microbiome of four species of butterflyfish: a bacterial diversity reservoir in coral reefs

Reverter, M., Sasal, P., Tapissier-Bontemps, N., Fagervold, S., Lecchini, D., Suzuki, M. Characterisation of the gill mucus microbiome of four species of butterflyfish: a bacterial diversity reservoir in coral reefs. To be submitted to FEMS Microbial Ecology (IF = 3.5).

Characterisation of the gill mucus microbiome of four species of butterflyfish: a bacterial diversity reservoir in coral reefs.

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Abstract

Native microbiota play an important role in gill homeostasis and prevention of infections, however very few studies have investigated the mucus microbiome of fish gills. We characterised the gill mucus microbiome of four butterflyfish species. Although the microbiome diversity of gill mucus varied significantly between species, Shannon diversities were surprisingly high ($H = 3.7 - 5.7$). We observed differences in the microbiome composition of the different butterflyfishes, with *Chaetodon lunulatus* and *C. ornatissimus* having the closest microbiomes, which differed significantly from *C. vagabundus* and *C. reticulatus*. The core microbiota was constituted mainly by Proteobacteria followed by Actinobacteria and Firmicutes. *C. lunulatus* and *C. ornatissimus* microbiomes were mostly dominated by Gammaproteobacteria with *Vibrio spp.* as the most abundant genus. *C. vagabundus* and *C. reticulatus* microbiomes presented similar abundances of Gammaproteobacteria and Alphaproteobacteria, which were well represented by *Acinetobacter spp.* and *Paracoccus spp.*, respectively. In conclusion, our results indicate that different fish species present bacterial specificities that might arise from bacterial-host coevolution and ecological parameters. Mucus layers are nutrient hotspots for heterotrophic bacteria living in oligotrophic environments. The high bacterial diversity found in butterflyfish gill mucus might indicate that external fish mucus surfaces might act as a reservoir for coral reef bacterial diversity.

Keywords: fish gill mucus, microbiome, butterflyfishes, coral reef fishes.

1. Introduction

The associations between metazoans and commensal microorganisms are among the most ancient and successful associations in nature (McFall-Ngai et al. 2013; Lowrey et al. 2015). Animals and their associated microorganisms share a common evolutionary history, since they have coevolved in response to environmental selective pressures over hundreds of millions of years (Zilber-Rosenberg and Rosenberg, 2008). Since host-microbiota interactions are linked to host physiology, maintaining microbiota homeostasis is a key factor to avoid pathogen proliferation and diseases (Kamada et al. 2013; Sommer and Bäckhed, 2013). Studies on human gut microbiota show that microbiota provide essential health benefits to its hosts, particularly by regulating immune homeostasis (Wu and Wu, 2012). In fish, disruption of microbiota homeostasis (dysbiosis) resulted in a decrease of probiotic-like bacteria and an increase in pathogenic bacteria (Boutin et al. 2013, 2014). Recent studies also show that bacterial isolates from fish gut and skin displayed antibacterial and antifungal activities against human and fish pathogens, suggesting a protective role of fish native microbiota against pathogens (Sanchez et al. 2012; Lowrey et al. 2015).

Fish external microbiota diversity (skin and gills) has been reported to be higher than internal (gut) microbiota (Smith et al. 2007; Lowrey et al. 2015). Fish microbiota seems to be species-specific and fish skin and gut microbiome is in most cases, significantly different than the surrounding water microbiome (Smith et al. 2007; Larsen et al. 2013, 2015). However, Giatsis et al. (2015) found that changes in water bacterial communities were highly correlated with changes in the gut of tilapia larvae. Similarly, And Mouchet et al. (2011) found that gut bacterial genetic diversity was significantly influenced by sampling site, both studies indicating that microorganisms in surrounding water might be a significant contributor to fish gut microbiomes.

To date, most studies on fish microbiota have investigated fish gut and skin microbiome, but gill microbiome diversity and their functional roles remain poorly understood (Larsen et al. 2013, 2014; Giatsis et al. 2015). Gills are the main respiratory organ in fish, and are composed of four pairs of vascularised gill arches with hundreds of gill filaments, which increase their contact surface for oxygen diffusion by folding into secondary lamella. In addition to respiration, gills perform other functions including osmoregulation, pH balance,

ammonia excretion, hormone regulation, detoxification and immune defence (Maina, 2002). Gills are constantly in contact with water, and therefore they are continuously exposed to pathogens (Xu et al. 2016). They are covered by a mucus layer that acts as the first physical and biochemical barrier against pathogens (Roberts and Powell, 2003). Gill mucus production increases following gill infection, and a recent study reported decreased oxygen diffusion and an increase of pathogenic bacteria in fish exposed to high sediment concentrations, impairing normal gill function (Ferguson et al. 1992; Hess et al. 2015). However, despite their importances in conserving gill homeostasis and preventing gill infection, native gill microbiota remain poorly studied.

In this study, we aimed to cast light on the gill microbiome of four sympatric butterflyfish species (*Chaetodon lunulatus*, *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) from Moorea (French Polynesia). Butterflyfishes (family Chaetodontidae) are emblematic and widely distributed coral reef fish species, which feed mostly but not exclusively on coral (Pratchett, 2005). Coral reefs are among the most productive and biologically diverse ecosystems on Earth, and despite the fact that coral microbiome has attracted increasing attention in recent years, most microbial communities of reef-associated organisms remain poorly studied (Ainsworth et al. 2015). The main objectives of this study were to characterise the bacterial diversity and taxonomic composition of gill mucus of four butterflyfishes and compare the microbiome variability between four sympatric species. This is one of the first studies evaluating the gill mucus microbiome and to the best of our knowledge it is the first comprehensive study of gill microbiome in coral reef fishes using high-throughput sequencing.

2. Materials and methods

2.1. Mucus sampling and DNA extraction

Four butterflyfish species (*C. lunulatus*, *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) were sampled (n = 20) on the island of Moorea (French Polynesia). Fish were spear-fished, placed in individual plastic bags with seawater and brought immediately to the laboratory for gill dissection. Gill mucus was carefully scraped with a sterile spatula into

sterile tubes. Mucus DNA was extracted right after collection using the DNeasy Blood & Tissue kit (QIAGEN, Courtaboeuf, France). Concentration of the nucleic acids was quantified by measuring the absorbance at 260 nm and their purity was checked by measuring the ratio of OD 260/280 nm and 230/260 nm. DNA samples were sent for 454 pyrosequencing by Mr. DNA (Shallowater, TX, USA) as previously described (Croué et al 2013). Briefly a PCR step (30 cycles) with a HotStart HiFidelity Polymerase and primers 28F (59 TTTGATCNTGGCTCAG 39) and 519R (59 GTNTTACNGCGGCKGCTG 39) was performed in order to amplify the hypervariable V1-V3 region of the 16S rRNA gene. Tag-encoded FLX amplicons were sequenced on the Roche 454 FLX sequencer using Titanium (Roche) reagents. Samples were identified by the fish initials and an order number (Ex. CL1-CL5 for *C. lunulatus* replicates)

2.2. Data treatment

Multiplex raw sff files were analysed using a hybrid analysis pipeline as previously described (Croué et al. 2013) with some modifications. In brief, denoising was done by AmpliconNoise V1.25 (Quince et al. 2011) implemented in Qiime V1.5 (Caporaso et al. 2010) with a small modification to allow it to be ran in an iDataplex (IBM) cluster. *De novo* chimera detection and removal was performed with the uchime module (Edgar et al. 2010; 2011) of usearch 5.2 (<http://drive5.com/usearch/>). Non-chimeric sequences were unweighted and grouped into operational taxonomic units (OTUs) with 98% sequence identity using the usearch V5.2 method implemented in Qiime V1.5. The longest sequences in the OTU were selected as representatives. OTUs were classified using the rdp_classifier software implemented in Qiime V1.5 and a database based on the Green genes August 2013 taxonomy (<http://greengenes.secondgenome.com>) modified to exclude orders, and to correct to comply to current official nomenclature [(list of prokaryotic names with standing in nomenclature (<http://www.bacterio.net>))]. Based on this classification, OTUs representing chloroplasts and mitochondria were removed. The resulting OTU table was further treated to remove (using custom *bash* and *awk* scripts) OTUs for which the representative sequence was shorter than 372 base pairs (400 bp minus the length of the primer), OTUs which were represented by a single sequence in the ensemble of samples and a “Root” taxonomy status by the rdp_classifier analysis, as well as sequences failing aligning by pynast aligner implemented in

Qiime. Finally, as one of the samples from *C. vagabundus* was clearly an outlier in preliminary analyses, the sample was removed from the OTU table along with all OTUs present exclusively in this sample, as was one OTU abundant in sample CV1 corresponding to a chloroplast, but not identified as such by the RDP classifier. Sequences were aligned and filtered (Lane, 1991) using *mothur v.1.38.0* and a neighbour joining tree constructed using *phylip v3.6a3*. Alpha-diversity indices (observed richness, Chao 1, Shannon and evenness) were calculated using an OTU table rarefied to 1499 sequences using the *single_rarefaction.py* function of *Qiime v1.5* summarised per fish species (mean and standard deviation). ANOVA and Tukey post-hoc test were used to detect significant differences in Shannon's diversity (H) between fish species. OTUs expected richness of fish species was computed using the *specaccum* function of the *vegan* package for R. Principal coordinates analysis (PCoA) using weighted unfrac distance, which takes in account both the OTUs abundance and the phylogenetic distance between the OTUs, was used to assess the differences between the microbiomes of the different fish species (Lozupone et al. 2011) using a relativized (percentage of reads per sample) OTU table. Permutational multivariate analysis of variance (PERMANOVA, function *adonis* of the *vegan* package for R) and pairwise comparisons between group levels with corrections for multiple testing (function *pairwise.perm.manova* of the *RVAideMemoire* package for R) were used to evaluate statistically significant differences of PCoA groups between fish species. The number of shared OTUs among all fish species combinations were calculated and represented using a Venn diagram (using the rarefied OTU table). Microbiome composition of the four butterflyfish species was characterised and results are displayed by bacterial phylum abundances. The bacterial families and genera with abundances higher than 5% at least in one of the samples were also identified.

3. Results

3.1. Microbiome diversity

A total of 1940 OTUs were obtained from the gill mucus of four butterflyfish species (*C. lunulatus*, *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*). Alpha-rarefaction indices

showed that one of the *C. vagabundus* species possessed a remarkably different microbiome (45% of its OTUs were exclusive of this sample, including 5 different phyla). Therefore, we considered it as an outlier and the following analyses included only four *C. vagabundus* samples (Figure 1. Supplementary information). After removal of the outlier sample, 1450 OTUs were obtained in the full OTU table and 1041 in the rarefied table.

Shannon diversity varied significantly between fish species, with the highest diversity found in *C. vagabundus* ($H = 5.7 \pm 0.5$) and the lowest in *C. reticulatus* ($H = 3.7 \pm 0.7$). *C. lunulatus* and *C. ornatissimus* presented similar diversities ($H = 4.7 \pm 1.2$ and $H = 4.8 \pm 0.5$ respectively). Microbial community evenness (E) was slightly higher in *C. vagabundus* ($E = 0.8 \pm 0.1$). Observed and expected richness were both the highest in *C. lunulatus* (142.8 ± 62.3 and 473 OTUs respectively) followed by *C. ornatissimus* (114.4 ± 38.9 and 370 OTUs respectively), *C. vagabundus* (137.0 ± 53.7 and 426 OTUs respectively) and *C. reticulatus* (45.6 ± 20.4 and 179 OTUs respectively) (Table 1).

	Chao 1	Shannon (H)	Evenness (E)	Observed richness	Expected richness
<i>C. lunulatus</i>	230.5 ± 99.7*	4.7 ± 1.2	0.6 ± 0.1	142.8 ± 62.3*	473
<i>C. ornatissimus</i>	181.0 ± 83.7	4.8 ± 0.5	0.7 ± 0.1	114.4 ± 38.9	370
<i>C. reticulatus</i>	48.5 ± 23.2*	3.7 ± 0.7*	0.7 ± 0.2	45.6 ± 20.4*	179
<i>C. vagabundus</i>	160.9 ± 75.3	5.7 ± 0.5*	0.8 ± 0.1	137.0 ± 53.7	426

Table 1. Alpha-diversity estimates for the four butterflyfish species (mean ± standard deviation). * indicates significant difference (p-value < 0.05).

3.2. Microbiota differences between butterflyfishes

Among the 1041 OTUs, only 27 OTUs were found in all four butterflyfishes. This “core microbiota” (27 OTUs) represented between 18 and 35% of all bacterial sequences in each fish species. The core microbiota (at the OTU level) consisted mainly of Proteobacteria, Actinobacteria and Firmicutes (class Clostridia) and one cyanobacteria OTU (Table 2). *C. lunulatus* was the species with the highest number of specific OTUs (273 OTUs), which represented between 5 and 19% of its bacterial community. *C. vagabundus* had the second highest number of specific OTUs (249 OTUs, 26-66% of all OTUs), followed by *C. ornatissimus* (143 OTUS, 6-15% of all OTUs), and finally *C. reticulatus*, with only 80

specific OTUs that represented between 16 and 62% of its bacterial community. *C. lunulatus* and *C. ornatissimus* shared the highest number of OTUs (total of 167 shared OTUs), while *C. lunulatus* and *C. reticulatus* shared the lowest (40 OTUs) (Figure 1).

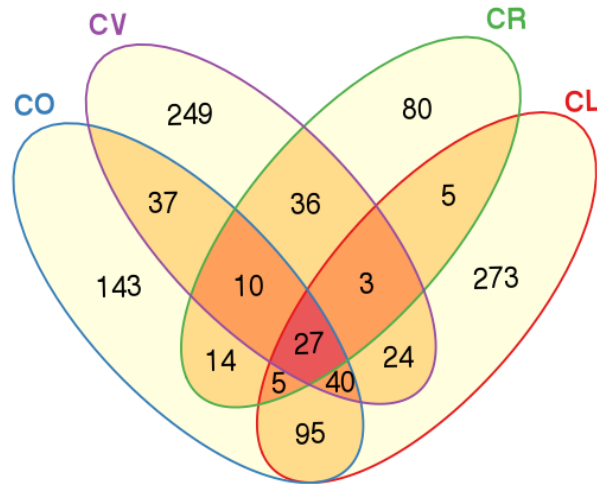


Figure 1. Venn diagram representing shared operational taxonomic units (OTUs) between fish species. CL = *C. lunulatus*, CO = *C. ornatissimus*, CR = *C. reticulatus* and CV = *C. vagabundus*.

OTUs	Phylum	Class	Family	Genus	Species
44, 68			Corynebacteriaceae	<i>Corynebacterium</i>	-
131	Actinobacteria	Actinobacteria	Micrococcaceae	<i>Micrococcus</i>	<i>luteus</i>
3			Propionibacteriaceae	<i>Propionibacterium</i>	<i>acnes</i>
114	Cyanobacteria	Synechococcophycideae	Synechococcaceae	<i>Synechococcus</i>	
1, 25, 36, 38, 47, 219, 4467	Firmicutes	Clostridia	-	-	-
58, 208			[Mogibacteriaceae]	-	-
280		Alphaproteobacteria	Rhodobacteraceae	<i>Nautella</i>	-
71, 269				<i>Ruegeria</i>	-
854		Betaproteobacteria	Burkholderiaceae	<i>Ralstonia</i>	-
696			-	-	-
79				<i>Acinetobacter</i>	-
42	Proteobacteria		Moraxellaceae	<i>Acinetobacter</i>	<i>venetianus</i>
142		Gammaproteobacteria		<i>Enhydrobacter</i>	-
67			Salinisphaeraceae	-	-
5				<i>Photobacterium</i>	-
0			Vibrionaceae	<i>Vibrio</i>	-
426				<i>Vibrio</i>	<i>shilonii</i>

Table 2. Core microbiota (OTUs and their classification) of the four butterflyfish species (*C. lunulatus*, *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*).

The Principal Coordinate Analysis (PCoA) based on weighted Unifrac distances showed significant microbiota differences between the pairs *C. lunulatus*/*C. ornatissimus* and *C. reticulatus*/*C. vagabundus* (p -value < 0.05, Adonis test and pairwise comparisons) (Figure 2).

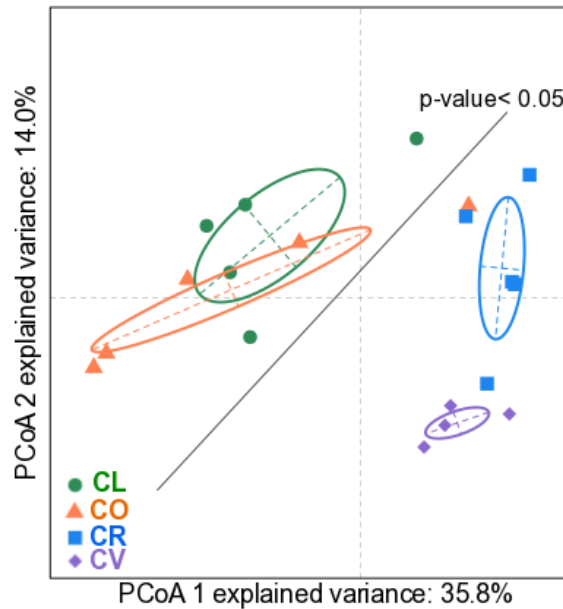


Figure 2. PCoA analyses of the gill mucus microbiota of four species of butterflyfish (CL = *C. lunulatus*, CO = *C. ornatissimus*, CR = *C. reticulatus* and CV = *C. vagabundus*) based on Weighted Unifrac distances. The Diagonal black line shows significant difference (p -value < 0.05) between *C. reticulatus*/*C. vagabundus* and *C. lunulatus*/*C. ornatissimus* tested by a PERMANOVA analysis and pairwise comparisons corrected for multiple testing.

3.3. Microbiome composition

We are fully aware that percentage of OTUs does not represent exactly the percentage of cellular abundances, and thus hereafter abundances refer to the percentage of total sequence reads in the final OTU table. Unidentified OTUs (Unassigned) represented less than 10% in all four butterflyfish species. Proteobacteria was the most abundant phylum in the four butterflyfish species. Gammaproteobacteria dominated the microbial assemblage of *C. lunulatus* and *C. ornatissimus* (90% and 80% of the total Proteobacteria), while Alphaproteobacteria was more abundant in *C. vagabundus* (59% of total Proteobacteria). *C. reticulatus*, whose community presented the highest abundance of Proteobacteria (67% of total OTUs), presented 41% of Alphaproteobacteria, 19% of Betaproteobacteria and 39% of Gammaproteobacteria. In addition to Proteobacteria, only Actinobacteria and Firmicutes were

present in all samples of butterflyfish gill mucus, being particularly well represented in *C. vagabundus*, *C. reticulatus* and *C. ornatissimus*. *C. lunulatus* and *C. ornatissimus* presented a higher abundance of Bacteroidetes (11% and 17% respectively). *C. lunulatus* also presented a notably higher abundance of Fusobacteria (4%) which was nearly absent in the other fish species (Figure 3).

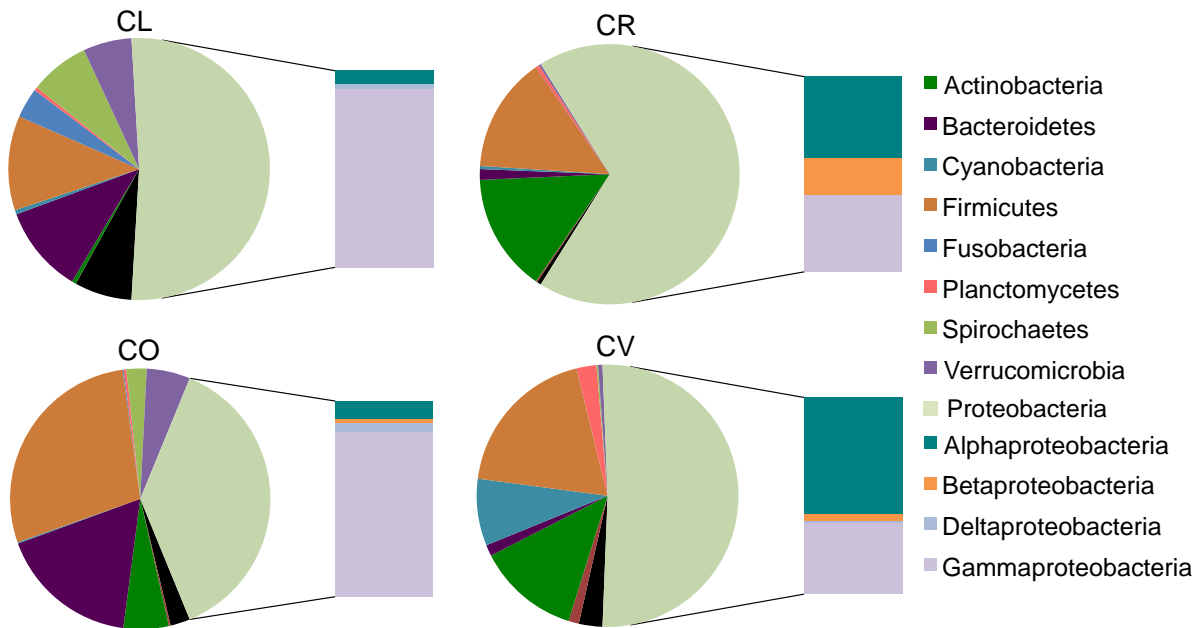


Figure 3. Bacterial diversity at the phylum level (pie chart) and class level (bars) determined by 454 pyrosequencing. Pie diagrams show the percentage of sequenced clones belonging to different bacterial phylum for each fish species analyzed. Bar graphs represent the percentage of Proteobacteria classes detected in each fish species. CL = *C. lunulatus*, CO = *C. ornatissimus*, CR = *C. reticulatus* and CV = *C. vagabundus*.

At the family level, we saw some consistent associations between some bacterial families and *Chaetodon* species, but also high inter-individual variation (Figure 4). As expected from the PCoA analysis, dominant families were most similar between *C. lunulatus* and *C. ornatissimus*, and *C. reticulatus* and *C. vagabundus*.

Groups with interesting associations with *C. lunulatus* and *C. ornatissimus* include 1) Vibrionaceae (mostly *Vibrio* spp.), generally the most abundant microbial taxa in *C. lunulatus* (between 3 and 90 %) and *C. ornatissimus* (between 1 and 41%) 2) Verrucomicrobiaceae (mostly *Akkermansia* spp.) with abundances up to 15% in *C. lunulatus* and *C. ornatissimus*,

3) Hahellaceae (mostly *Endozoicomonas* spp.), which were frequent in *C. lunulatus* and *C. ornatissimus*, reaching an abundance of 25% in one *C. lunulatus* individual, while absent in *C. reticulatus* samples, 4) Spirochaetaceae (*Spirochaeta* spp.) with abundances ranging from 1 to 10% in *C. lunulatus* and *C. ornatissimus*, and rarely present in *C. vagabundus* and totally absent in *C. reticulatus* and 5) Ruminococcaceae (genus undetermined), which represented around 5% of the total bacterial assemblage of *C. lunulatus* and *C. ornatissimus*, but they were absent in *C. reticulatus*.

Groups displaying interesting associations with *C. reticulatus* and *C. vagabundus* include 1) Moraxellaceae (mainly *Acinetobacter* spp.), which were more abundant in *C. reticulatus* and *C. vagabundus* than *C. lunulatus* and *C. ornatissimus* 2) Propionibacteriaceae (mostly *Propionibacterium* spp.) with abundances of 2 - 8% in *C. reticulatus* and 0.5 – 12% in *C. vagabundus* and 3) Corynebacteriaceae (genus *Corynebacterium*), which represented around 10% of the microbiota community in most *C. reticulatus* and *C. vagabundus* samples while were nearly absent in all *C. lunulatus* and *C. ornatissimus*.

A number of bacterial families appeared to have been particularly associated to a single Chaetoron species. Anaplasmataceae were surprisingly abundant (70 and 33%) in two samples of *C. reticulatus*, and were mainly constituted by OTUs belonging to the *Neorickettsia* genus. Burkholderiaceae (genus *Ralstonia*) were also remarkably more frequent in *C. reticulatus* (up to 25%) than in the other fish species. Rhodobacteraceae were present in all fish species, but were particularly abundant in *C. vagabundus*, where they reached abundances up to 32%. As were “Exiguobacteraceae”, mainly the *Exiguobacterium* genus, nearly specific to *C. vagabundus*, displaying abundances lower than 1% in the other fish species. Finally, Fusobacteriaceae (genus undetermined) had abundances in *C. lunulatus* ranging from 0.4 to 7.1%, while they were nearly absent in all other three fish species.

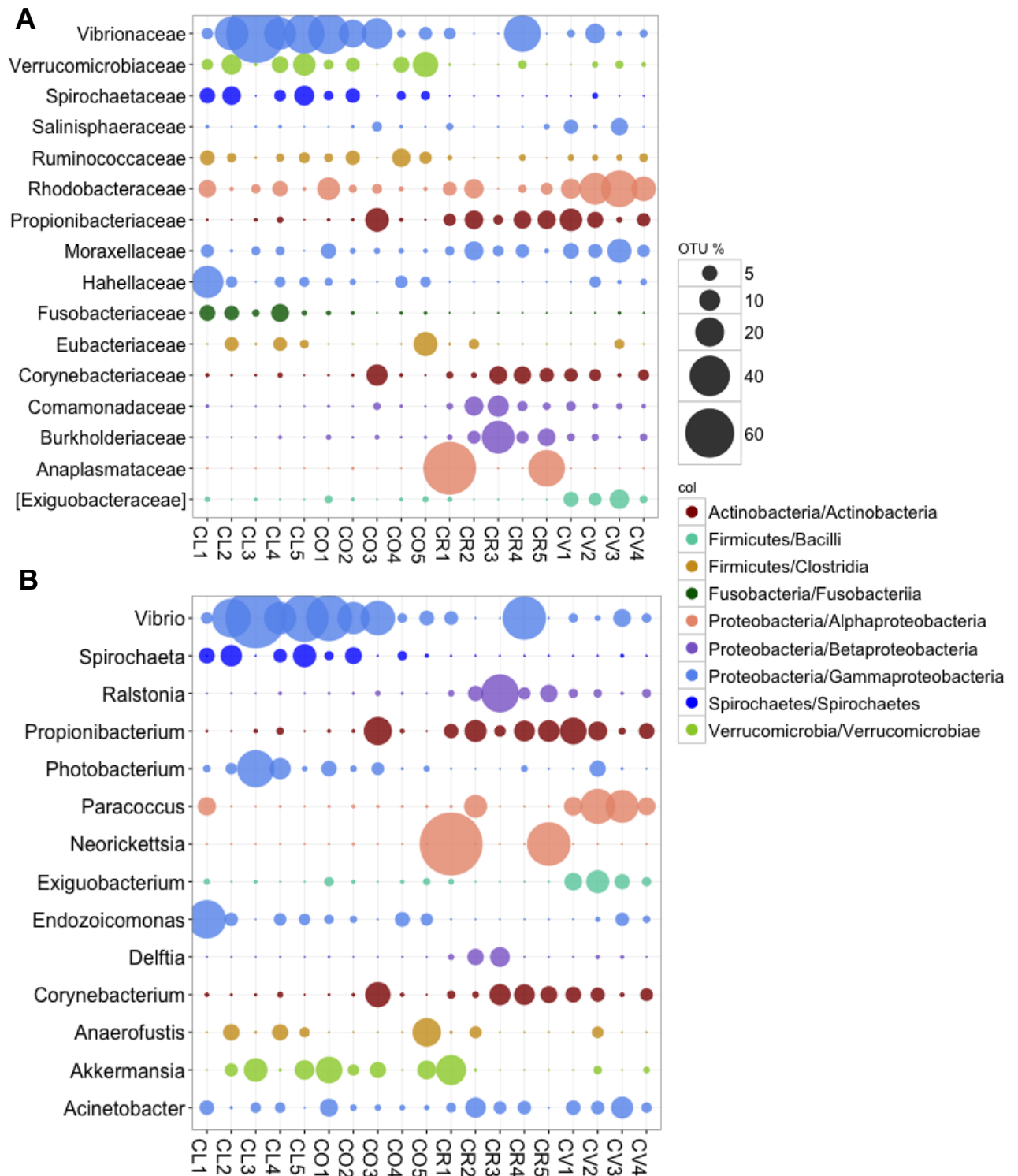


Figure 4. Abundant taxa a) family and b) genus of the gill mucus microbiome. Only taxa with abundance $\geq 5\%$ at least in two of the samples is plotted. CL = *C. lunulatus*, CO = *C. ornatissimus*, CR = *C. reticulatus* and CV = *C. vagabundus*.

4. Discussion

Gills are an essential organ to fish well-being, thus studying their normal microbiota is of great importance to understand their role in gill homeostasis and prevention of infections. Fish gill mucus is in constant contact with the environment, proportioning a rich nutrient habitat for heterotrophic bacteria, compared to the surrounding oligotrophic coral reef waters. In this study we characterized and compared the gill mucus microbiome of four sympatric coral reef fish species of Moorea (French Polynesia) using tag 454-pyrosequencing. Although the microbiome diversity of gill mucus of butterflyfishes varied significantly between species, Shannon diversities were surprisingly and consistently high ($H = 4.8 - 5.7$, 26 phyla, with the exception of *C. reticulatus* that displayed a significant lower diversity $H = 3.7$) when compared to gill microbiome of cultured rainbow trout (*Oncorhynchus mykiss*) ($H < 4.5$, 11 phylum), gut microbiota of surgeonfishes ($H = 0.5 - 3.9$), tropical sponges ($H = 1.6 - 4.3$), some coral species ($H = 0.8 - 2.0$), coral reef water ($H = 0.6$) or coral reef sediment ($H = 0.8$) (Bayer et al. 2013; Moitinho-Silva et al. 2014; Lowrey et al. 2015; Miyake et al. 2015; Glasl et al. 2016). Although these higher levels could be partly attributed to the higher OTU cutoff (98%) used in our study as well as the primers used for amplification. Nonetheless regarding the difference to rainbow trout, it is to be expected that the microbiome of cultured fish would be less diverse than wild fish, which have higher genetic variability as well as higher dietary plasticity (Givens et al. 2015). We would also like to note that higher Shannon diversity from other coral species (*Montastrea faeolata*) has also been reported ($H = 6.5$) (Kimes et al. 2010). Interestingly, while *C. vagabundus* presented the most diverse (Shannon's diversity) bacterial community, *C. lunulatus* presented the highest OTU richness, highest Chao1 index, and highest number of specific OTUs, indicating that *C. lunulatus* has a large number of rare OTUs that contribute little to the overall bacterial community, or conversely that *C. vagabundus* has a more even bacterial community.

Although all fish species were collected at the same sampling site, they presented significant microbiome differences. While we saw high inter-individual variation, by comparing at least 4 individual microbiomes per species, we were able to highlight some general trends regarding the *Chaetodon* microbiomes. *C. lunulatus* and *C. ornatissimus* were the two species clearly with the most similar microbiomes. These two species are the closest phylogenetic relatives and they are both obligate corallivores (Pratchett, 2005; Fessler and

Wesneat, 2007). In contrast, *C. vagabundus* is an omnivorous species that belong to a different phylogenetic clade (Pratchett, 2005; Fessler and Wesneat, 2007). Therefore, a similarity between their gill microbiomes is not unexpected and these results suggest that gill mucus microbiome of fishes might be influenced together by phylogeny and/or ecological characteristics of the host species. On the other hand, we cannot explain why *C. reticulatus*, which is ecologically and phylogenetically closer to *C. lunulatus* and *C. ornatissimus*, presents a microbiome that is more similar to that of *C. vagabundus*. Overall, our results show that gill mucus microbiota is somewhat host-specific, however more studies should assess variability of gill mucus microbiome over space and time to confirm the associations presented here.

The microbiome associated to the gill mucus of the four butterflyfish species were dominated by Proteobacteria, which is in accordance with previous studies on the gill microbiome of rainbow trout and the skin and gut microbiome of several fish species (Sullam et al. 2012; Larsen et al. 2015; Lowrey et al. 2015). Gammaproteobacteria represented over 80% of the total Proteobacteria in *C. lunulatus* and *C. ornatissimus* gill mucus, which was mainly represented by families Vibrionaceae, Pseudomonaceae and Hahellaceae bacteria. High abundances of *Vibrio* and *Photobacterium* are reported in normal microbiota of fish skin, (Smith et al. 2007; Yücel and Balci, 2010; Larsen et al. 2015) but to date only one study has reported a high incidence of *Vibrio spp.* in the gills of marine fish used for human consumption (Yücel and Balci, 2010). *Vibrio* species are widespread in the marine environment and are also known to be associated to coral mucus and tissues and in some cases responsible for coral disease (reviewed in Rosemberg et al 2007). Unfortunately, it is very difficult if not impossible to discriminate between *Vibrio* species based on 16S rRNA sequences alone, so we prefer not to speculate the origin or the role of the OTUs associated with, *C. lunulatus* and *C. ornatissimus* gill mucus (Sawabe et al. 2013). However, the fact that *Vibrionaceae* are less abundant in *C. reticulatus* and *C. vagabundus*, suggest that these bacteria are not just passively transferred from the corals or surrounding seawater, but can be in fact have a stronger association to these species. Endozoicomonas species are often found as part of the corals holobiont or in close association to other marine invertebrates (e.g. bivalves, sea slugs; Kurahashi et al. 2007; Bayer et al. 2013; Hyun et al. 2014), and interestingly a recent study also identified Endozoicomonas strain (Katharios et al. 2015)

pathogenic to fish, but with a sequence quite distant to the *Endozoicomonas* found in our samples.

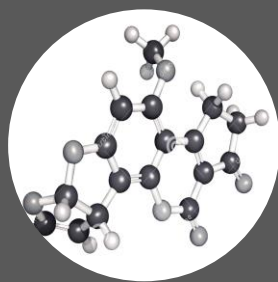
Verrucomicrobia species, which were abundant in *C. lunulatus* and *C. ornatissimus* (1 – 15%, mainly an *Akkermansia* unidentified species) have been found in low abundances in fish guts (0.3 - 1%), fish skin and coral tissues (1 - 3%) (Kooperman et al. 2007; Chiarello et al. 2015; Miyake et al. 2015; Lawler et al. 2016). Verrucomicrobia are ubiquitous (although rarely in very high proportions) in the marine environment, both in the water column and sediment, but little is known of the functional role they might play in association with organisms such as fish (Freitas et al. 2012).

The nearly specific presence of Fusobacteria in *C. lunulatus* was also remarkable, especially considering that this fish species has the particular trait of not being infected by gill monogenean parasites. Fusobacteria are anaerobic bacteria found in large quantities in fish gut (Clements et al. 2014), however to the best of our knowledge this is the first record of Fusobacteria in external surfaces of fish. Fusobacteria are known to produce a short-chain fatty acid, butyrate, which is the end product of fermentation of carbohydrates including those found in mucins (Bennett and Eley, 1993). In mammals, butyrate provides many benefits to the host, enhancing mucus production and acting as an anti-carcinogen and anti-inflammatory (von Engelhardt et al. 1998; Andoh et al. 1999). Some Fusobacteria species such as *Cetobacterium somerae*, which has an identical sequence to an OTU present in the gill mucus of *C. lunulatus*, is also known to produce abundant amounts of vitamin B12 (Merrifield and Rodiles, 2015).

C. reticulatus and *C. vagabundus* had a much higher proportion of Alphaproteobacteria, that corresponded mostly to higher abundances in Rhodobacteraceae (mainly *Paracoccus*) and remarkable abundances of *Neorickettsia* in two samples of *C. reticulatus*. *Paracoccus* has been previously identified in fish skin, fish gut and corals (Sheu et al. 2011; Liu et al. 2013; Larsen et al. 2015). *Neorickettsia* species are normally intracellular pathogens that cause severe illnesses in mammals and are transmitted by flukes (Platyhelminthes: Digenea) that infect fishes (Vaughan et al., 2012). A potential new species of *Neorickettsia* has been identified as species specific from skin microbiota of striped mullet (*Mugil cephalus*), however their role and importance in fish microbiota remains unknown (Larsen et al. 2015).

Actinobacteria and Firmicutes, which seem to constitute the fish core microbiota along with Proteobacteria, were abundant in gill mucus of all fish species, especially in *C. reticulatus* and *C. vagabundus*. Interestingly, 10% of the microbial communities of *C. vagabundus* and *C. reticulatus* were composed of *Corynebacterium*, a pathogenic bacteria which was found to increase in gill mucus of clownfish exposed to high concentrations of sediment (Hess et al. 2015). Finally, we found remarkable high abundances of *Propionibacterium* and *Ralstonia* (2 - 8% and 1 - 25% respectively), which have been described as widespread coral rare associate species, which are closely associated to coral dinoflagellate symbionts, but undetectable on water surrounding corals (Ainsworth et al. 2015).

In conclusion, we found a high bacterial diversity in gill mucus of butterflyfishes, many of which are also found in the coral holobiont. Mucus layers are nutrient hotspots for marine heterotrophic bacteria living in oligotrophic environments such as coral reefs. Therefore, we hypothesise that external fish mucus surfaces can act as a reservoir for coral reef bacterial diversity. Our study shows that different fish species possess different microbiomes, indicating the presence of species specificities in some bacterial OTUs that might arise from bacterial-host coevolution and ecological parameters. However, there is a large part of the bacterial community (50%) that is common in the four fish species studied. We propose that there might be a bacterial transfer between sympatric organisms that could include other organisms such as corals. Bacterial chemotaxis has been shown between coral associate bacteria (e.g. Endozoicomonaceae, Rhodobacteraceae and Vibrionaceae) and coral mucus amino acids (Tout et al. 2015). However, fish mucus is known to contain a wide array of proteins, including the same amino acids found in coral mucus, therefore there might exist also a bacterial chemotaxis between natural occurring coral reef bacteria and coral reef fish mucus (Valdenegro-Vega et al. 2014). Finally, we have observed the presence of several potentially pathogenic bacteria in the gill mucus of healthy butterflyfishes. We suggest that a high bacterial diversity could control proliferation of pathogenic agents by direct competition as it has been proposed for the gut microbiota (Kamada et al. 2013). However, in stress conditions, a microbiota dysbiosis could lead to proliferation of pathogens and disease outbreaks such as shown by Hess et al. (2015).



CHAPTER V

Metabolomics reveals coral reef fish history traits

Reverter, M., Sasal, P., Banaigs, B., Lecchini, D., Lecellier, G., Tapissier-Bontemps, N., Metabolomics reveals coral reef fish history traits. *Coral reefs*, *submitted*. (IF = 3.0)

Fish mucus metabolomics reveals fish life history traits.

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Abstract

Fish mucus has important biological and ecological roles such as defense against fish pathogens and chemical mediation between several species. A non-targeted LC-MS metabolomic approach was developed to study gill mucus of eight butterflyfish species in Moorea (French Polynesia), and the influence of several fish traits (site of collection *i.e.* geographic site and reef habitat, species taxonomy, phylogeny, diet and parasitism levels) on the metabolic variability was investigated. A biphasic extraction yielding two fractions (polar and apolar) was used. Fish diet (obligate corallivorous, facultative corallivorous or omnivorous) arose as the main driver of the metabolic differences in the gill mucus in both fractions, accounting for 23% of the observed metabolic variability in the apolar fraction and 13% in the polar fraction. Level of monogenean gill parasites was the second most important factor shaping the gill mucus metabolome, and it explained 10% of the metabolic variability in the polar fraction and 5% in the apolar fraction. A multiple regression tree revealed that the metabolic variability due to parasitism in the polar fraction was mainly due to differences between non-parasitized and parasitized fish. Phylogeny and butterflyfish species were factors contributing significantly to the metabolic variability of the apolar fraction (10% and 3% respectively) but had a less pronounced effect in the polar fraction. Finally, geographic site and reef habitat of butterflyfish species did not influence the gill mucus metabolome of butterflyfishes.

Keywords: fish mucus, metabolomics, coral reefs, butterflyfishes.

1. Introduction

Ecosystems are dynamic assemblies of organisms that interact with each other in multiple ways, forming complex networks of ecological interactions (Olf et al. 2009). Chemically mediated interactions either within one species (intraspecific) or between different species (interspecific) are by far, the most widespread interactions in the living world. Chemical cues determine species foraging behavior, competitive interactions, commensal associations, pathogenic invasions and selection of mates and habitats (Hay, 2009). Metabolic diversity is associated to ecosystem complexity, where a high biodiversity and the associated multiple ecological interactions would maintain and promote diversity of metabolites (Moore et al. 2014). Metabolite production is highly dependent on environmental factors such as temperature, light and nutrients, being very susceptible to biotic and abiotic stresses such as nearby competitor's pressure (Kooke and Keurentjes, 2011; Lages et al. 2012; de Caralt et al. 2013).

The mucosal layers covering fish epidermis and gills are the main surface of exchange between fish and the surrounding environment (Shepard 1993). Fish mucus presents numerous biological and ecological roles including respiration, osmotic regulation, excretion, defense against pathogens and chemical mediation between species (Purcell and Anderson, 1995; Brinchmann, 2016). Therefore, mucosal surfaces have a high functional importance reflecting both fish physiology and responses to environment. Mucus is mainly constituted of glycoproteins, called mucins, but it also contains immune-related molecules such as lysozyme, immunoglobulins, lectins and antimicrobial peptides, lipids and carbohydrates (Brinchmann et al. 2016). Smaller molecules composition of fish mucus has been much less studied, but some toxins (e.g. tetrodotoxin) or mycosporine-like aminoacids, which are acquired through diet, have been found in fish skin mucus (Eckes et al. 2008; Noguchi and Arakawa 2008). Most studies on fish mucus have focused on one or few molecules at a time, giving a fragmented idea on fish mucus composition. Recent advances in omics technologies, however, have allowed studying fish mucosal surfaces using more global approaches (Brinchmann et al. 2016). For example, the use of proteomics, which gives a detailed protein composition, revealed a high diversity of immune competent molecules previously unreported in fish mucus (Rajan et al. 2011; Cordero et al. 2015). Metabolomics, which is the comprehensive study of metabolites, is one of the most recent "omics" sciences (Sumner et

al., 2003). Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002). A major benefit of metabolomics is the simultaneous analysis of multiple metabolites that do not necessarily need to be known, providing a powerful tool to analyze chemical diversity and changes in the natural environment (Kusano et al. 2015). Metabolomic approaches have proven useful to distinguish among individual signals and thus might serve in biomarker discovery in a wide range of topics, from disease biomarkers to indicators of short-term environmental changes in ecology or even in chemotaxonomy studies (Wolfender et al. 2009). A recent study investigated for the first time the fish mucus metabolome and found over 200 distinct metabolites in fish skin mucus, some of which showed altered levels in response to bisphenol A exposure (Ekman et al. 2015).

Coral reefs are among the most productive and biologically diverse ecosystems on Earth. However, anthropogenic activities such as overfishing and pollution and rapid climate change compromise their persistence (Pandolfi et al. 2011). Continued loss of coral cover and the erosion of the complex habitat structure associated with healthy coral reefs are expected to have significant consequences for a vast number of reef-associated species such as butterflyfishes (Munday et al. 2008). Butterflyfishes (family Chaetodontidae) are a diverse and conspicuous family of coral reef fishes distributed widely in all tropical seas. Butterflyfish ecology and behavior have been extensively studied, and although they can consume a variety of prey, including algae, polychaetes, crustaceans and coral, most species feed primarily if not exclusively on scleractinian corals (Pratchett, 2005).

The present work aimed to study butterflyfishes gill mucus metabolome and explore the relationship between fish mucus metabolome, fish life history and the environment in which butterflyfish live. Understanding which fish traits affect the mucus metabolome is the first step towards the comprehension of the fish mucus roles and its dynamics. Besides, since fish mucus is in direct contact with the environment, it can provide useful information about both the surrounding environment and the fish ecological interactions. A non-targeted LC-MS metabolomic approach was developed to study fish gill mucus. The influence of several fish traits (site of collection, species taxonomy, diet, parasitism level, and phylogeny) on the metabolic variability was investigated. Metabolic profiles of gill mucus of eight Chaetodon species, belonging to three phylogenetic clades and displaying contrasting diet preferences

and levels of parasitism were studied at three geographic sites in Moorea Island (French Polynesia) and at different reef habitats (fringing reef, barrier reef, outer slope).

2. Material and methods

2.1. Sampling and chemical extraction

Eight butterflyfish species (*C. auriga*, *C. lunulatus*, *C. lunula*, *C. ornatissimus*, *C. quadrimaculatus*, *C. reticulatus*, *C. vagabundus* and *C. ulietensis*) (n = 110) were sampled at three geographic locations in Moorea (French Polynesia). In one of the locations (Ta'ahiamanu), fish were sampled in the different reef habitats: fringing reef, barrier reef and outer reef slope (Figure 1).

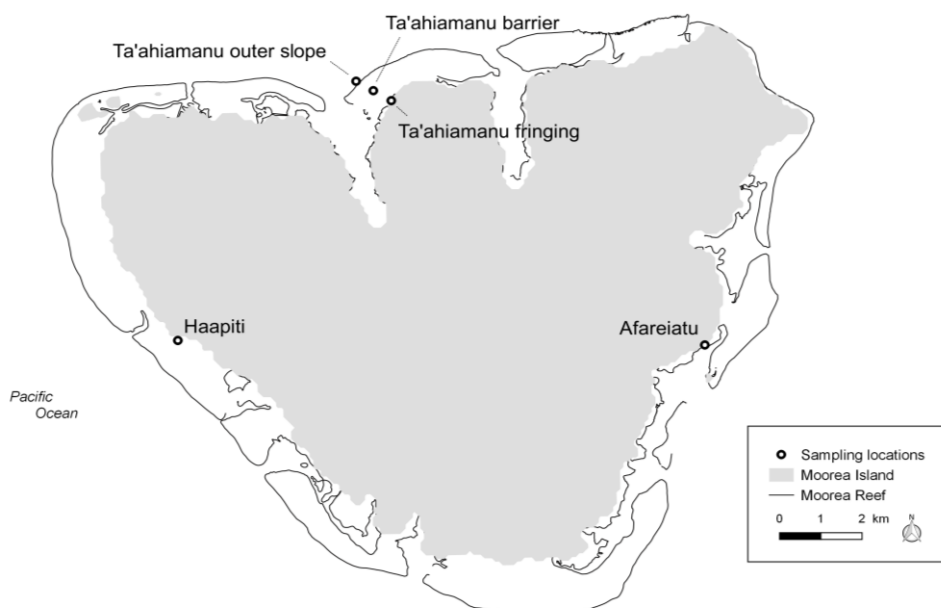


Figure 1. Map of the Moorea island (French Polynesia) with the sampling sites at three geographic locations (Ta'ahiamanu, Haapiti and Afareiatu) and three reef environments (fringing reef, barrier reef and outer slope) in the Ta'ahiamanu site.

Fish were spear-fished, put in individual plastic bags with seawater and brought to the laboratory as soon as possible for dissection. Gill mucus was collected by carefully scraping with a sterile spatula into sterile tubes and was freeze-dried and kept at -20°C until extraction.

10 mg of the freeze-dried samples were extracted using a two-step biphasic extraction, yielding two separate fractions, where polar and apolar metabolites could be extracted simultaneously (Wu et al. 2008). Samples were suspended in 1.5 mL of H₂O/MeOH (2:0.5) and vortexed, and then 1.5 mL of MeOH/CH₂Cl₂ (2.5:2) was added. The mixture was extracted 15 min in an ultrasonic bath and then centrifuged 20 min at 3500 G (4°C). The two fractions were carefully collected with glass pipettes into separate tubes. The apolar fraction was dried under nitrogen flux, and the polar fraction was freeze-dried. Polar and apolar fractions were resuspended in 300 µL MeOH and filtered through a 20 µm polytetrafluoroethylene (PTFE) filter. The quality control (QC) samples were prepared by pooling small aliquots from the polar and apolar extracts, respectively.

2.2. LC-MS analysis

LC-MS analyses were performed with a LC-DAD-ESI-MS system from ThermoScientific (MA, USA) equipped with an Accela PDA detector and a LCQ Fleet 2300 mass spectrometer equipped with an electrospray ionization source. The equipment was controlled, and the data was handled by the Excalibur Data Software. For chromatographic separation a Kinetex C6-Phenyl analytical column (100 × 2.10 mm, 2.6 µm particular size; Phenomenex, CA, USA) equipped with the corresponding pre-column was used with H₂O (A) and acetonitrile (B) as carriers, both containing 0.1 % v/v formic acid. The flow rate was 500 µL/min, column temperature was maintained at 30 °C and injection volume was 10 µL. For the polar fraction a linear biphasic gradient from 2 to 100 % acetonitrile in water was applied over a period of 30 min, followed by 10 min washing and 15 min re-equilibration with 2% of B before the next injection. For the apolar fraction a linear gradient was conducted from 30% to 70% of B in 5 min, then from 70% to 80% of B in 20 min and from 80% to 100% of B in 5 min, followed by a 10 min wash with 100% of B and 15 min re-equilibration with 30 % of B before the next injection. Full scan data were acquired in positive ionization mode for both fractions. The mass spectrometer analyzer parameters were set as follows: sheath gas flow rate 25 a.u., aux gas flow rate 5 a.u., sweep gas flow rate 1 a.u., capillary temperature 275 °C, capillary voltage +2 V, tube lens voltage 120 V and spray voltage 4 kV and a full scan mass window of 130–2000 m/z. To randomize analytical sequences and reduce systematic error associated with instrumental drift, a Latin square was carried out for each of

the sample sequences (polar and apolar). QC samples were analyzed at the beginning, the end and equidistantly throughout the sequence. In order to detect column contamination throughout the sequence methanol blank samples were analyzed just before each QC sample.

2.3. Data treatment and statistical analysis

LC-MS raw data files were converted to netcdf files with the Excalibur Software. Netcdf files were processed using the R package Xcms to detect, deconvolute and align features (molecular entities with a unique m/z and a specific retention time). Xcms analysis of these data provided a matrix containing the retention time, m/z value and integrated peak area of the identified features. Data were normalized by log transformation prior to statistical analysis.

Data were analyzed considering the following fish traits: species taxonomy, collection site (reef habitat and geographic site), diet, phylogeny and level of parasitism. Butterflyfish were classified upon their diets as obligate corallivores (*C. lunulatus*, *C. ornatissimus* and *C. reticulatus*), facultative corallivores (*C. quadrimaculatus* and *C. lunula*) and omnivorous (*C. auriga* and *C. vagabundus* and *C. ulietensis*) following *in situ* observations in Moorea and existing bibliography (Pratchett, 2005). Phylogenetic groups were determined from the phylogenetic tree in Fessler and Wesneat (2007) and described as follows: 2 = *Chaetodon* clade 2 (*C. quadrimaculatus*); 3 = *Chaetodon* clade 3 (*C. lunulatus*, *C. ornatissimus*, *C. reticulatus*); 4 = *Chaetodon* clade 4 (*C. auriga*, *C. vagabundus*, *C. lunula* and *C. ulietensis*).

Butterflyfishes were classified in three groups depending on the gill parasitism intensities of dactylogyrids (Platyhelminthes, Monogenea) described in Reverter et al. (2016): 1 = non parasitized (*C. lunulatus*), 2 = low parasitism intensity (*C. auriga*, *C. lunula* and *C. ulietensis*), 3 = high parasitism intensity (*C. ornatissimus*, *C. quadrimaculatus*, *C. reticulatus* and *C. vagabundus*).

Metabolomic diversity of each sample was determined by the Shannon's diversity index (H), and differences across fish traits were tested using the analysis of variance (ANOVA) and the Tukey's HSD test of an ANOVA. Permutational multivariate analysis of variance using distance matrices (PERMANOVA) (adonis function, package vegan for the R software) was used to identify the significant fish traits linked to the metabolic variability in gill mucus of butterflyfishes in polar and apolar fractions. Principal component analysis (PCA) was then

used to visualize the gill metabolic variation on the significant fish traits (determined with the PERMANOVA analysis) and the 10 most important contributors (features) were represented. Multiple regression trees (MRT, package *mvpart* for the R software) were used to obtain further details of the fish traits explaining the chemical variability in both fractions. MRT allow to rank fish traits according to their contribution, by producing a tree where at each split data is partitioned into two mutually exclusive groups, each of which is as homogeneous as possible, displaying this way the major factors determining the variability (De'ath, 2002; Vignon and Sasal, 2010). Only the fish traits with a contribution higher than 0.1% were plotted.

2.4. Technical vs. biological variance

Two types of variance should be considered in metabolic studies: the technical variance due to variation of sample preparation and instrumental analysis and the biological variance, existing between the analyzed samples. To study the biological variance, the technical variance must be lower. Biological variance was monitored by extracting and analyzing 5 different mucus samples from the fish species *C. lunula*. Technical variance (repeatability) was assessed by analyzing 5 independently prepared extracts (technical replicates) from a pool constituted of the same 5 samples of *C. lunula*. Samples were extracted and analyzed as described before (including the randomized analytical sequences and the QC). The two datasets (polar and apolar) were then pre-treated and statistical analyses were used to determine technical and biological variance.

3. Results

3.1. Technical vs. biological variance

Technical variability due to extraction procedure, analytical analysis and data processing was significantly smaller than biological variability. PCA revealed close clustering between technical replicates, while biological replicates were significantly more dispersed in both fractions (Figure 2). These results show the suitability of the metabolomic approach used for

the study of metabolic differences between different biological samples of butterflyfish gill mucus.

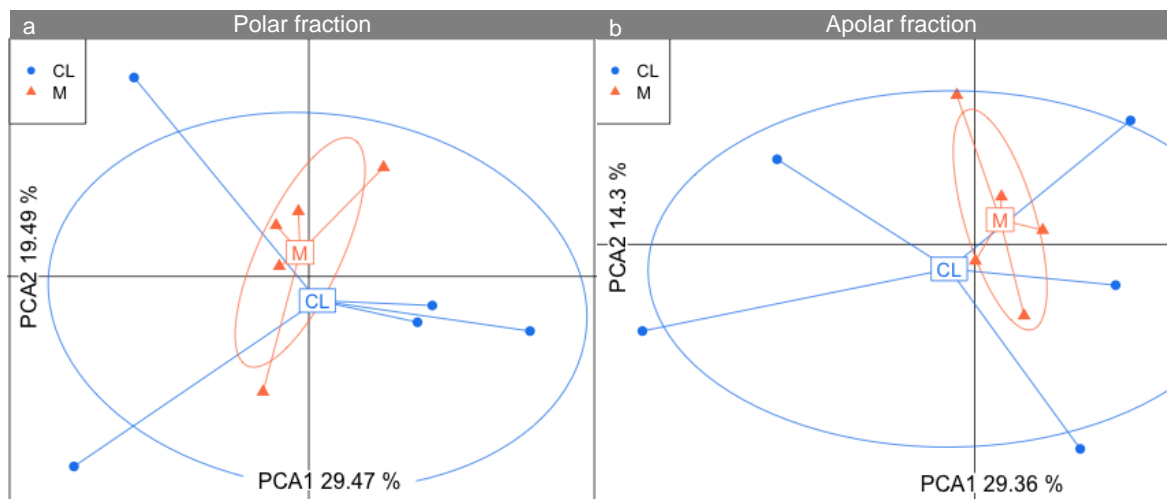


Figure 2. PCA plots of the technical (M) and biological variability (CL) for the polar (a) and apolar (b) fractions.

3.2. Metabolomic diversity

Metabolomic diversity (Shannon's index, H) calculated according to the analyzed fish traits (fish species, reef habitat, geographic site, phylogeny, diet and intensity of parasitism) showed no significant differences ($P > 0.05$) in the polar fraction. However, significant differences in the metabolomic diversity were found in the apolar fraction depending on the fish species, phylogeny, diet and level of parasitism (Figure 3). *C. ulietensis*, *C. lunula* and *C. vagabundus* had significant higher metabolomic diversity ($P < 0.001$) than *C. lunulatus* and *C. ornatissimus*. *C. ulietensis* and *C. lunula* also presented a higher metabolomic diversity ($P < 0.001$) than *C. reticulatus*. Butterflyfish from the clade 4 had higher metabolomic diversity than clade 3. Obligate corallivores presented significantly ($P < 0.001$) lower metabolomic diversity than facultative corallivores and omnivores. Fish never parasitized presented also lower metabolomic diversity than fish with low levels of parasites ($P < 0.001$) (Figure 3).

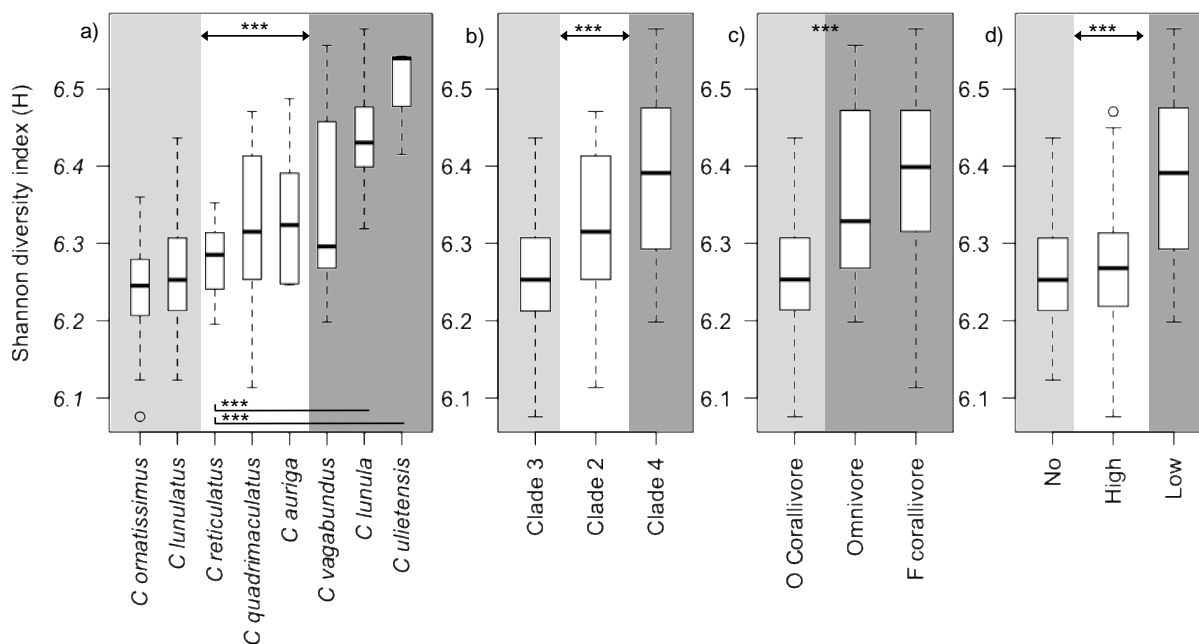


Figure 3. Shannon's diversity indices of the fish mucus metabolome according to the different factors analyzed. a) butterflyfish species, b) phylogenetic clades, c) diet, O corallivore = obligate corallivore, F corallivore = facultative corallivore and d) intensity of parasitism, No = never parasitized, High = high parasitism intensities, Low = low parasitism intensities. Statistical significance was determined using the Tukey's test of one-way analysis of variance, *** $p = 0.001$. Light grey and dark grey regions indicate statistically significant differences between categories.

3.3. Metabolic variability of gill mucus

We conducted a PERMANOVA analysis on distance matrices to identify the fish traits that contributed significantly to the metabolic variability of the two fractions of gill mucus extract. Geographic site (Afareiatu, Ta'haiamanu and Haapiti) and reef habitat (fringing reef, barrier reef and outer slope) did not affect significantly the metabolic profiles in any of the gill mucus fractions (polar and apolar). Butterflyfish species played a significant role in the metabolic variability of the apolar fraction, explaining 10% of the variability observed. Phylogeny, diet and parasitism were significant factors in both fractions. Diet explained most of the metabolic variability observed in both fractions (23% of the apolar fraction and 13% of the polar fraction), followed by parasitism (5% of the apolar fraction and 10% of the polar fraction). Phylogeny explained 3% of the metabolic variability in both fractions (Table 1). PERMANOVA analysis shows that studied variables (species, geographic site, reef

environment, diet and parasitism) only partly explain the metabolic variability (42% of the apolar fraction and 30 % of the polar fraction).

Parameter	Fraction	F	R2	P - value
Species	Apolar	3.55	0.08	< 0.001
	Polar	0.84	0.01	0.517
Geographic site	Apolar	1.17	0.01	0.26
	Polar	2.48	0.03	0.07
Reef environment	Apolar	2.34	0.02	0.25
	Polar	1.94	0.03	0.06
Phylogeny	Apolar	6.29	0.03	< 0.001
	Polar	4.09	0.03	0.02
Diet	Apolar	19.79	0.23	< 0.001
	Polar	7.28	0.13	< 0.001
Parasitism	Apolar	7.96	0.05	< 0.001
	Polar	8.61	0.10	< 0.001

Table 1. Results of the PERMANOVA test explaining the metabolic variability of gill mucus of butterflyfishes.

To visualize the significant sources of gill mucus metabolite variation and their relative distribution, we conducted PCA analysis for both polar (Figure 3) and apolar (Figure 4) fractions. The ten most important contributors of the polar fraction discriminate diagonally (bottom left - top right) the fish traits (Figure 3), whereas the ten most important contributors of the apolar fraction discriminate the fish traits on the top left – bottom right diagonal (Figure 4).

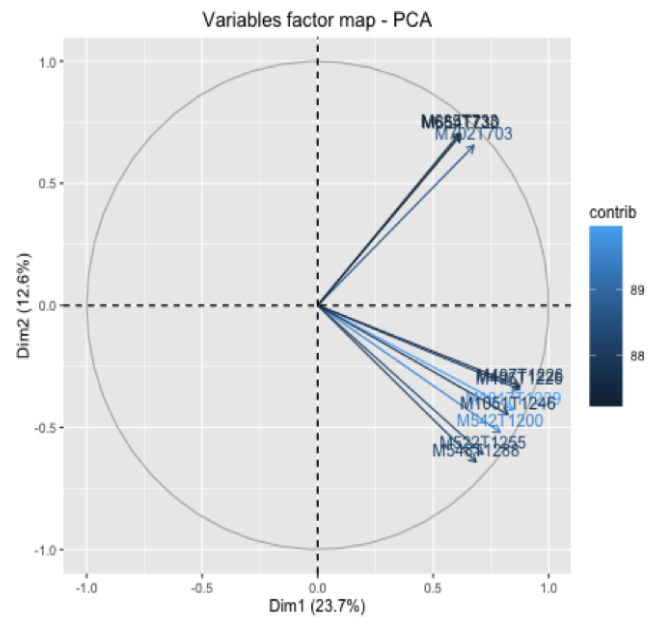
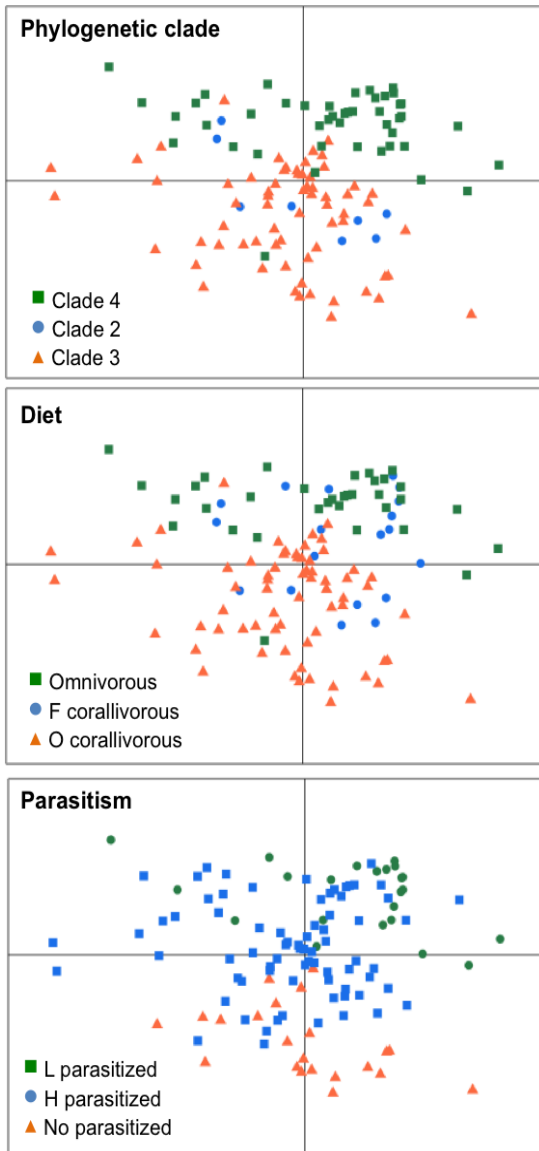


Figure 3. PCA of gill mucus metabolome of the polar fraction with distribution of the samples on the first two components identified to the significant fish traits (fish phylogenetic clade, fish diet and fish level of parasitism). The ten most important contributors are represented on the circle of correlation in the right graph.

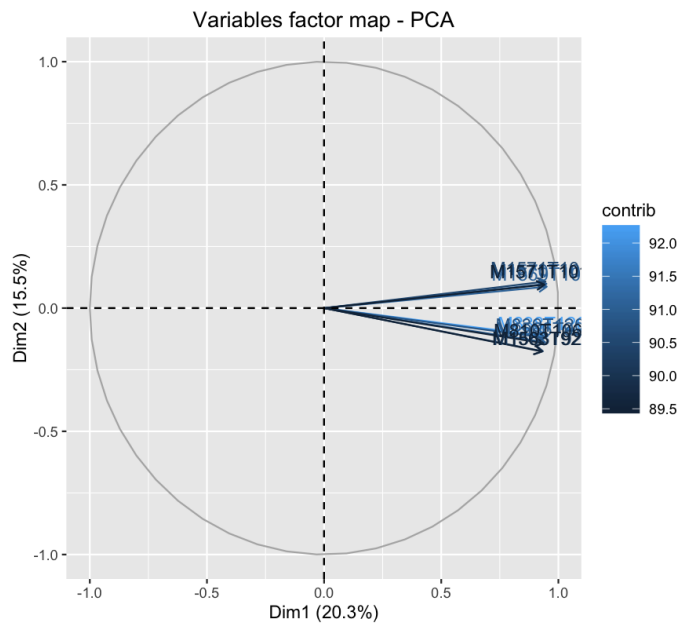
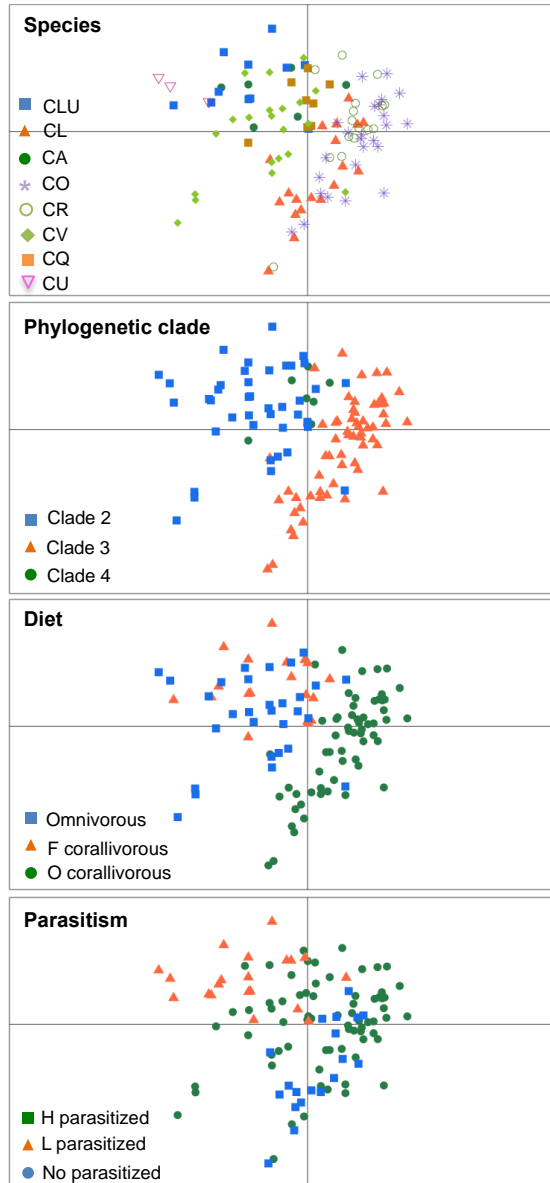


Figure 4. PCA of gill mucus metabolome of the apolar fraction with distribution of the sample on the first two components identified to significant fish traits (fish species, fish phylogenetic clade, fish diet and fish level of parasitism). The ten most important contributors to the observed PCA analysis are represented on the circle of correlation in the right graph.

Multiple regression tree analysis also shows that diet, parasitism and less importantly phylogeny are the main factors shaping the metabolomic differences in the butterflyfish gill mucus. In the polar fraction the main metabolomic differences are due to differences between obligate corallivores/omnivores and facultative corallivores, followed by differences between parasitized fish (high and low intensities) and non-parasitized fish (7.4% out of the 10% of metabolic variability explained by parasitism). Metabolomic diversity in parasitized fish is explained by differences between obligate corallivore fish and omnivores (Figure 5a).

The main factor driving metabolomic variability in the apolar fraction is the difference between facultative corallivores/omnivores and obligate corallivores. Differences in phylogeny (clade 2/4 and clade 3) explain the metabolic variability within the facultative corallivore and omnivorous fish. While, parasitism (non-parasitized vs. highly parasitized) explains the metabolic differences of obligate corallivorous fish (Figure 5b).

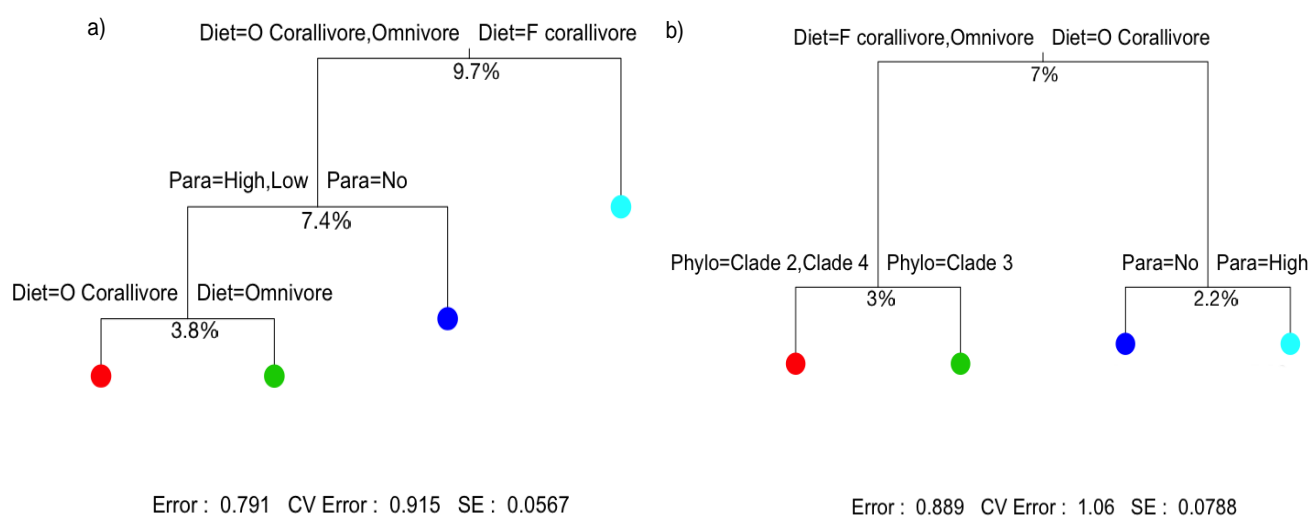


Figure 5. MRT of the gill mucus metabolome from the polar (a) and apolar (b) fractions with the % of variance explained by each split. O corallivore = obligate corallivore, F corallivore = facultative corallivore, High = high parasite intensities, Low = low parasite intensities, No = never parasitized.

4. Discussion

Mucus is involved in many regulatory processes in fish, playing a major role in both fish physiology and fish environmental interactions (Brinchmann, 2016). This study at Moorea, is the first attempt to study the gill mucus metabolome and the factors that might explain some of its variability. Improving the knowledge on fish mucus constituents and dynamics will cast light on some biological and ecological roles of fish mucus, as well as help future research dealing with fish mucus variations.

Distinct metabolite profiles are often observed between different species of similar organisms, and sometimes these metabolic species-specific signatures have been proposed as chemotaxonomic markers (Ivanisevic et al. 2010; Sogin et al. 2014). In our study, different metabolic fingerprints between species of butterflyfish were observed in the apolar fraction, but no significant differences were observed in the polar fraction (Figure 4), which indicates that apolar metabolites are species-specific. However, other factors such as diet seemed to play a bigger role in the metabolite fingerprints than butterflyfish species (Figure 7). Butterflyfish species are phylogenetically close, so our results could indicate that in historically close complex organisms (such as fish) other factors than species could have a major impact in the metabolic profiles.

Spatial variations in metabolic fingerprints have been observed for a number of species, including soft corals (Matlock et al. 1999; Rohde et al. 2012; He et al. 2014). Our results show that metabolic fingerprints of butterflyfish mucus did not vary significantly over the three geographic sites studied or between different reef habitats (Figure 5). Although there are certain spatial differences in the reefs of Moorea, most of the environmental properties (temperature, salinity and pH) are quite homogeneous (Lamy et al. 2015). We thus hypothesize that since butterflyfish gill mucus metabolome responds to ecological drivers, spatial variations might be observed in environmentally different geographic locations, but they could be masked in similar environments.

Differences in metabolic profiles in fish internal organs like liver have been observed in cultured fish after the administration of different diets (Abro et al. 2014; Silva et al. 2014). Different food intake can stimulate contrasted physiological reactions in fish, which result in the differential production of metabolites in fish internal and external organs or tissues

(Roosta et al. 2014, Silva et al. 2014). For example, Ghehdarijani et al. (2016) found that fish fed with a supplemented diet in garlic displayed higher protein levels and alkaline phosphatase in fish skin mucus. Besides, feeding on chemically defended organisms (such as algae, corals or sponges) can result in dietary sequestration of secondary metabolites (Pennings and Paul, 1993; Thoms et al. 2003). Our results show for the first time the importance of fish diet in the chemical composition of gill mucus. Butterflyfish feed on a wide variety of invertebrates, thus we can imagine that different diet preferences such as scleractinian corals, algae or polychaetes will expose butterflyfish to different secondary metabolites, that could significantly alter fish mucus chemical composition by 1) affecting fish physiology differently, for example depending on the toxicity of the secondary metabolites ingested and the detoxifying pathways developed (McLean and Duncan, 2006) or 2) assimilating some of the secondary metabolites for defense purposes (Thoms et al. 2003). Butterflyfish with broader diets (omnivorous and facultative corallivores) had a significant higher metabolic diversity than obligate corallivorous fish, indicating that gill metabolome reflects the diversity of food intake. Trophic transfer of metabolites and their accumulation in external fish mucus has been observed before, such as the UV-absorbing molecules (MAAs), which are selectively sequestered via trophic transfer from corals, or tetrodotoxin, which is also accumulated through the food chain (Eckes et al. 2008; Noguchi and Arakawa 2008).

Fish mucus also possesses a complex community of commensal microorganisms that play an important role in maintaining mucus homeostasis and protection against pathogens (Boutin et al. 2013; Llewellyn et al. 2014). A recent study showed that some mucus bacteria are able to produce bioactive secondary metabolites, which could contribute to the overall mucus metabolome (Sanchez et al. 2012). Different fish foraging behaviors could affect the mucus microbiome by encountering different bacterial communities or by benefiting some bacterial populations more than others, and this could be reflected in the mucus metabolome (Antwis et al. 2014).

The loss of coral reef resilience is often cryptic, and it has been hard to characterize the instability of coral reef ecosystems before their collapse (Bellwood et al. 2004). When coral reefs fail to regenerate after natural and human impacts they undergo shifts to alternate states where coral dominance is often replaced by algae dominance (Hughes, 1994; Bellwood et al. 2004). Butterflyfishes are closely related to coral cover, and since their mucus reflects directly their feeding habits we could suppose that butterflyfish mucus metabolome might prove

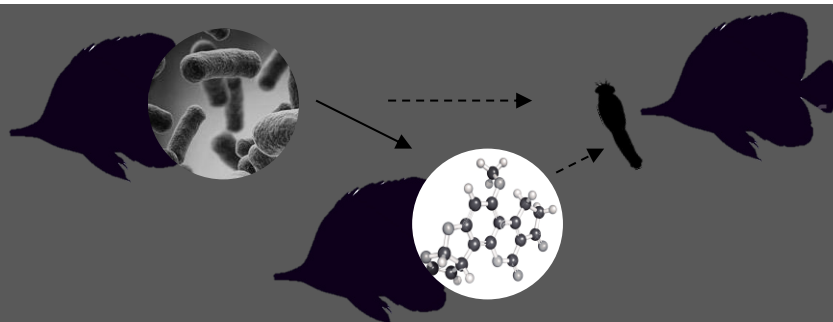
useful detecting changes in coral reefs. Decreases in coral cover might induce some facultative corallivorous feeders to adapt to the available food resources and such changes might be reflected in the mucus metabolome (Wen et al. 2016). Selective feeding of butterflyfish on coral lesions associated to infective diseases where there is a high density of pathogenic organisms absent in healthy coral tissues could also produce remarkable differences in the fish mucus metabolome (Frias-Lopez et al. 2003; Chong-Seng et al. 2011). Some recent studies have shown that metabolic profiles of corals and their symbiotic dinoflagellates change under temperature stress (Klueter et al. 2015; Sogin et al. 2016). Therefore, changes in the metabolic profiles of butterflyfish preys are also likely to infer variations in butterflyfish mucus metabolome.

Parasitism also influences the gill mucus metabolome of butterflyfishes, especially in the polar fraction. There are several possible explanations for the relationship between the mucus metabolome and gill parasitism 1) fish mucus molecules could be responsible for the presence or absence of parasites, either by the presence of antiparasitic molecules or by the presence of recognition molecules that would enhance parasitism (Kallert et al. 2011), 2) parasites can modulate the production of certain fish mucus molecules and 3) metabolic differences arise from fish physiological answers to parasitism.

Parasites are often more sensible to environmental degradation than their hosts (Marcogliese, 2005). Thus habitat modification and environmental degradation would have major impacts in the parasite communities, with a decrease in parasite richness and an increase in the abundance of some virulent parasites (Tylianakis et al. 2007; Johnson et al. 2013). Dactylogyrids are the primarily parasites of butterflyfish gills and we have observed they significantly influence our results revealed that their presence is related with the gill metabolome of butterflyfishes. Therefore, changes in dactylogyrid communities and intensities due to coral reef degradation and perturbations are likely to be observed in the mucus metabolome.

To the best of our knowledge, this is the first study investigating the gill mucus metabolome. We have developed a non-targeted LC/MS metabolomic approach to study gill mucus metabolome of butterflyfishes and have studied how several butterflyfish life traits influence metabolic variability. Diet appeared as the major determinant in the differences observed among butterflyfish mucus samples followed by level of dactylogyrid parasites.

Butterflyfish phylogeny and species were also responsible for some of the metabolic variability observed. Our results show that fish mucus reflects simultaneously several life traits of their hosts and could potentially be used as indicator of the environmental status of their host habitat. Metabolomics is a powerful tool to compare the biological response of organisms to several conditions and perturbations. Mucus is the first surface of exchange between fish and its environment, and therefore the analysis of the mucus metabolome offers a unique opportunity to study organism responses to environmental disturbances which might allow an early detection of environment degradation, before the known macroecology indicators take place (e.g. loss of fish richness - abundance, increase in seaweed cover) (Heenan and Williams, 2013). This is the first study exploring coral reef fish mucus metabolome and some of the drivers of its variability, but an important part of the variability remains unexplained. We think that maybe other factors not considered in this study like fish age, gender or exact health status of the fish could also play a role in the metabolic variability of fish mucus.



CHAPTER VI

Do mucus microbiome and metabolome interact to prevent ectoparasitic infection in a coral reef fish?

Do mucus microbiome and metabolome interact to prevent ectoparasitic infection in a coral reef fish?

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Abstract

Chaetodon lunulatus is a butterflyfish that unlike other sympatric and phylogenetically close butterflyfishes with similar ecologies, has never been found parasitized in the gills by monogenean Platyhelminthes. In order to find a possible explanation for this intriguing phenomenon we first tested whether *C. lunulatus* mucus would be deterrent to these parasites in an *in vivo* study. *C. lunulatus* gill mucus induced a significant decrease in the number of parasites in three highly parasitized *Chaetodon* species. We followed this *in vivo* study by characterizing the chemical composition of the mucus in conjunction to a microbiome analysis in order to better understand the factors leading to parasite deterrence in this butterflyfish species. The microbiome of *C. lunulatus* contained higher relative abundances of Fusobacteria, Spirochaetes and Vibrionaceae when compared to other *Chaetodon* species. These bacteria were strongly positively correlated with an overexpression of β -hemoglobin peptides in *C. lunulatus*, suggesting that might exist a relation between them. As hemoglobin peptides are known to present a wide array of bioactivities, we hypothesize that they might play a role in preventing monogenean infection of *C. lunulatus*. However, further evaluation of the biological activities of purified peptides will be performed in the laboratory to confirm the previous hypotheses. This is one of the first studies where fish metabolome and microbiome has been related to fish parasitism. However, more research is needed to clarify the exact role of these bacteria and peptides to the absence of parasites in *C. lunulatus*.

Keywords: fish mucus, metabolomics, microbiome, coral reefs, butterflyfishes, parasitism.

1. Introduction

Parasites modify competitive and trophic interactions between organisms, playing a major role in community and ecosystem organisation (Hudson et al. 2006). However, environmental degradation due to pollution, and climate change or culture conditions such as overcrowding can lead to parasite outbreaks and high host mortalities (Leung et al. 2013; Fox et al. 2015).

Host-parasite interactions, which result from a tight host-parasite co-evolution, are extremely complex (Quigley et al. 2012; Song et al. 2015). Hosts develop defence mechanisms to avoid parasite infections, and parasites develop counter-adaptations to overcome these defence mechanisms (Schmid-Hempel, 2008). Host defence mechanisms include physical barriers such as epithelial surfaces or mucous membranes and innate and adaptive immune responses (Janeway et al. 2001, Magnadottir, 2006).

The mucosal layers are the first protective barrier against pathogens, avoiding parasite contact with epithelial cells and promoting their clearance (Hansson et al. 2012). Mucus is mainly constituted of glycoproteins but it also contains a wide array of innate immune molecules such as lysozyme, immunoglobulins, lectins and numerous antimicrobial peptides (Gómez et al. 2013). Antimicrobial peptides (AMPs), which can either be derived from a biologically inactive proprotein that is processed to the active form or derived from larger, functional proteins, display biological activities against numerous pathogens including bacteria, fungi and metazoan parasites (Vizioli and Salzet, 2002; Radek and Gallo, 2007). Several fish AMPs with antiparasitic activities (e.g. piscidins and Hb β P-1) have been isolated from different tissues of cultured fish including gill epithelium and epidermal mucus (Colorni et al. 2008; Ullal et al. 2008, 2010).

Native microbiota have multiple effects on hosts, such as modulation of the host innate immune system, which can influence disease resistance and parasitic infections (Gomez and Balcazar, 2008; Cirimotich et al. 2011a, 2011b). For example, Cirimotich et al. (2011a) found that production of reactive oxygen species (ROS) by mosquito endogenous bacteria inhibited the development of malaria *Plasmodium* parasites. However, relationships between vertebrate microbiota and parasite co-occurrence are poorly understood (Bär et al. 2015; Kreisinger et al.

2015; Reynolds and Finlay, 2015). In fish, some studies have highlighted the importance of fish mucus microbiota as protection against opportunistic bacteria (Boutin et al. 2013), but to our knowledge no study has yet studied the interaction between fish microbiome and parasite infection.

Butterflyfishes (family Chaetodontidae) are a diverse and emblematic family of coral reef fishes distributed widely in all tropical seas. Butterflyfish phylogeny, ecology and behaviour have been extensively studied, and they are mostly territorial monogamous species that can consume a variety of prey including algae, polychaetes, crustaceans and coral (Pratchett, 2005). Butterflyfishes are naturally parasitized by monogeneans belonging to the family Dactylogyridae, which are direct cycle flatworms that attach to fish gills by two pair of hooks (Kearn, 1994; Plaisance et al. 2004). In a recent study we found that out of 34 butterflyfish species, only *Chaetodon lunulatus* was never parasitized by gill monogeneans (Reverter et al. 2016). This species does not present any remarkable different ecological or behavioural traits, therefore we hypothesised that specific parasitism differences between closely related butterflyfishes might arise from gill or gill mucus characteristics.

This study aims to investigate the role of *C. lunulatus* gill mucus in the absence of gill monogenean parasites in this fish species. We have performed an *in vivo* essay to test whether *C. lunulatus* gill mucus decreased monogenean rates on three highly parasitized butterflyfishes. Then, we studied the gill mucus microbiome and metabolome of several parasitized butterflyfishes versus the non-parasitised *C. lunulatus*. We found a higher abundance of strictly anaerobic bacteria (mostly Fusobacteria and sulfate-reducing bacteria) and *Vibrio* bacteria in *C. lunulatus*, which are positively correlated with overexpressed haemoglobin-derived peptides in this latter species. We suggest that *C. lunulatus* haemoglobin-derived peptides might display biological activities and might prevent ectoparasitic infection in *C. lunulatus*. This is the first study, to the best of our knowledge, where a systems biology approach combining metabolomics and microbiomics was used to study host-parasite interactions in fish.

2. Material and methods

2.1. In vivo test

Butterflyfishes (*C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) were collected alive at night using a hand-net. They were immediately placed and transported to the laboratory in a 10 L aerated bucket and placed in flow through 200 L tanks with a water renewal of 20 L/h and aeration via an airstone. After 36 h of acclimatisation, fish were anesthetized for 1 min with 0.1% of clove oil (*Syzygium aromaticum*). Five fish of each species were carefully injected 1 mL of freshly collected *C. lunulatus* mucus on the gill arcs. Control fish (n = 5 for each species) were anesthetized and handled as the treated fish, but without actually injecting mucus on the gill arcs. Treated fish and control fish were then placed into two different 200 L tanks. 24 h after the treatment, all fish were euthanised, gills were dissected and monogenean parasites were counted under a binocular microscope.

2.2. Fish mucus sampling

Eight butterflyfish species (*C. auriga*, *C. lunulatus*, *C. lunula*, *C. ornatissimus*, *C. quadrimaculatus*, *C. reticulatus*, *C. ulietensis* and *C. vagabundus*) were sampled (n = 110) on the island of Moorea (French Polynesia). Fish were spear fished, put in individual plastic bags with seawater and brought immediately to the laboratory for dissection, where gills were aseptically dissected. Gill mucus was collected by carefully scraping with a sterile spatula into sterile tubes. Mucus for microbiome analyses was submitted for DNA extraction right after collection. Mucus for metabolomics analyses was freeze-dried and kept at -80°C until chemical extraction.

2.3. Gill mucus microbiome

Microbiome of five species of butterflyfish (*C. lunulatus*, *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) were studied (n = 5 for each fish species). Mucus DNA was extracted using the DNeasy Blood & Tissue kit from QIAGEN (Courtaboeuf, France). Concentration of the nucleic acids was quantified by measuring the absorbance at 260 nm and their purity was

checked by measuring the ratio of OD 260/280 nm and 230/260 nm. DNA samples were sent for 454 pyrosequencing by Mr. DNA (Shallowater, TX, USA) as previously described (Croué et al 2013). Briefly a PCR step (30 cycles) with a HotStart HiFidelity Polymerase and primers 28F (59 TTTGATCNTGGCTCAG 39) and 519R (59 GTNTTACNGCGGCKGCTG 39) was performed in order to amplify the hypervariable V1-V3 region of the 16S rRNA gene. Tag-encoded FLX amplicons were sequenced on the Roche 454 FLX sequencer using Titanium (Roche) reagents.

2.3.1. Data treatment

Multiplex raw sff files were analysed using a hybrid analysis pipeline as previously described (Croué et al) with some modifications. In brief, denoising was done by AmpliconNoise V1.25 (Quince et al. 2011) implemented in Qiime V1.5 (Caporaso et al. 2010) and *de novo* chimera detection and removal was performed with the uchime module (Edgar et al. 2010 ; 2011) of usearch 5.2 (<http://drive5.com/usearch/>). Non-chimeric sequences were unweighted and grouped into operational taxonomic units (OTUs) with 98% sequence identity using the usearch V5.2 method implemented in Qiime V1.5. The longest sequences in the OTU were selected as representatives. OTUs were classified using the rdp_classifier software implemented in Qiime V1.5 and a database based on the Green genes August 2013 taxonomy (<http://greengenes.secondgenome.com>) modified to exclude orders, and to correct to comply to current official nomenclature [(list of prokaryotic names with standing in nomenclature (<http://www.bacterio.net>))]. Based on this classification, OTUS representing chloroplasts and mitochondria were removed. The resulting OTU table was further treated to remove (using custom *bash* and *awk* scripts) OTUs for which the representative sequence was shorter than 372 base pairs (400 bp minus the length of the primer), OTUs which were represented by a single sequence in the ensemble of samples and a “Root” taxonomy status by the rdp_classifier analysis, as well as sequences not alignable by pynast.

2.3.2. Data statistical analysis

Microbiome differences between parasitized and non-parasitized fish were assessed on the classified OTUs at family level, since it there was a considerable amount of OTUs not identified to genus and species level. Principal component analysis (PCA) was used to assess

the microbiome differences between the sample groups (parasitized vs. non-parasitized) in an unbiased manner and detect patterns (package *ade4* for the R software). Permutational Multivariate Analysis of Variance (PERMANOVA, function *adonis*, package *vegan* for R) was used to test significance of PCA group separations. Partial least squares discriminant analysis (PLS-DA), a form of supervised multivariate analysis that aims to find the maximum separation between a priori groups was performed (package *mixOmics* for R). PLS-DA model accuracy was calculated using a two-fold cross validation (2CV) and the resulting number of misclassifications (NMC) was compared to its permuted null distribution (999 permutations) to test for model significance (p -value < 0.05). To identify OTUs driving separation in the PLS-DA model, Variables Importance in Projection (VIP) were obtained (package *RVAideMemoire* for R). OTUs with a VIP score higher than 1.5 were retained and Kruskal-Wallis test was used to identify significant abundance differences of OTUs between the groups (parasitized and non-parasitized).

2.4. Metabolomics analyses

2.4.1. Chemical extraction

2.4.1.1. Chemical extraction for untargeted metabolomics analysis

10 mg of the freeze-dried samples were extracted using a two-step biphasic extraction, yielding two separate fractions, where polar and apolar metabolites could be extracted simultaneously (Wu et al. 2008). Samples were suspended in 1.5 mL of H₂O/MeOH (2:0.5), and then 1.5 mL of MeOH/CH₂Cl₂ (2.5:2) was added. The mixture was extracted 15 min in an ultrasonic bath and then centrifuged for 20 min at 3500 G (4°C). The two fractions were carefully collected with glass pipettes into separate tubes. The apolar fraction was then dried under nitrogen flux, and the polar fraction was freeze-dried. Polar and apolar extracts were re-suspended in 0.3 mL MeOH and filtered through a 20 µm polytetrafluoroethylene (PTFE) filter (Phenomenex, CA, USA). The quality control (QC) samples were prepared by pooling small aliquots from the polar and apolar extracts, respectively.

2.4.1.2. Chemical extraction of antimicrobial peptides

An acidic extraction was performed to enrich the extract in peptides following the method described in Vidal-Dupiol et al (2011). Briefly, 10 mg of freeze-dried mucus were re-

suspended in 1 mL of 2M acetic acid. The suspension was placed in a 4°C water bath, sonicated (Vibra- cell™ 75185, 50% power, three pulses of 30 s) and centrifuged at 10,000 x g for 20 min at 4°C, to remove the cellular fragments. The supernatant was immediately collected and prefractionated using a Strata C18 cartridge (500 mg/ 6 mL; Phenomenex, CA, USA). Briefly, the C18 column was washed using acidified water with TFA (0.05%), and three successive elutions were performed with 10, 60, and 80% acetonitrile in acidified water. The fractions obtained were lyophilised and reconstituted with 1 mL of a H₂O/acetonitrile (1:1) mixture.

2.4.2. LC-MS analysis

2.4.2.1. LC-MS analysis of the metabolomic sequence

LC-MS analyses were performed with a LC-DAD-ESI-MS system from ThermoScientific (MA, USA) equipped with an Accela PDA detector and a LCQ Fleet 2300 mass spectrometer. The equipment was controlled, and the data was handled by the Excalibur Data Software. LC elution conditions consisted of two eluants A (water with 1% formic acid) and B (acetonitrile with 1% formic acid) with a flow rate of 0.5 mL.min⁻¹, and an injection volume of 10 µL. The elution profile of the polar extract consisted of a linear gradient from 2% to 100% of B in 30 min followed by 10 min washing and 15 min re-equilibration with 2% of B before the next injection. For the apolar fraction a linear gradient was conducted from 30% to 70% of B in 5 min, then from 70% to 80% of B in 20 min and from 80% to 100% of B in 5 min, followed by a 10 min wash with 100% of B and 15 min re-equilibration with 30 % of B before the next injection. Full scan data were acquired in positive ionization mode for both fractions. The column used for both extracts was a Kinetex C6-Phenyl analytical column (100 × 2.10 mm, 2.6 µm particular size; Phenomenex, CA, USA) equipped with the corresponding pre-column with a fixed temperature of 30 °C. The mass spectrometer analyzer parameters were set as follows: sheath gas flow rate 25 a.u., aux gas flow rate 5 a.u., sweep gas flow rate 1 a.u., capillary temperature 275 °C, capillary voltage +2 V, tube lens voltage 120 V and spray voltage 4 kV and a full scan mass window of 130–2000 m/z.

To randomise analytical sequences and reduce systematic error associated with instrumental drift, a Latin square was carried out for each of the sample sequences (polar and apolar). QC samples were analyzed at the beginning, the end and equidistantly throughout the

sequence. In order to detect column contamination throughout the sequence methanol blank samples were analysed just each the QC sample.

2.4.2.2. UHPLC-HRMS/MS

Ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-HRMS/MS) analyses were performed on selected samples of non-parasitized fish (aqueous fractions and acidic extractions) in order to obtain a higher mass accuracy of the discriminant compounds obtained by the metabolomic analyses. Analyses were performed on a Dionex UltiMate 3000 UHPLC system (Thermo Scientific) equipped with a Corona® Veo detector and interfaced with a hybrid quadrupole Orbitrap mass spectrometer (Q Exactive, Thermo Scientific). The equipment was controlled by the Chromeleon 7.2 Software, and the data were handled using the Excalibur Data Software. Elution conditions, elution profile and column were the same as described for the polar extract on the LC-DAD-ESI-MS analyses. Mass spectrometric data were collected in continuum full-scan mode from m/z 150 – 2000 in positive mode with a resolution of 70,000 and a normalised collision energy (NCE) of 30. CID (Low-energy collisional dissociation) fragmentation was used to obtain MS/MS spectra for the selected ions ($RT_1 = 14.10-14.40$ min/ $M_1 = 644.8725$ Da ; $RT_2 = 15.00-15.50$ min/ $M_2 = 657.3557$ Da; $RT_3 = 15.40-15.70$ min/ $M_3 = 782.4199$ Da) with a resolution of 35,000 and a NCE of 35.

2.4.3. Data pre-treatment and statistical analysis

LC-MS raw data files were converted to netcdf files with the Excalibur Software. Netcdf files were processed using the R package XCMS to detect, deconvolute and align features (molecular entities with a unique m/z and a specific retention time). XCMS analysis of these data provided a matrix containing the retention time, m/z value and integrated peak area of the identified features. Data were normalised by log transformation prior to statistical analysis.

The gill mucus metabolome data was analysed using the same pipeline than the microbiomics data (section 2.3.2). Briefly, a PCA was performed to observe metabolite differences between the sample groups (parasitized and non-parasitized), and a PERMANOVA test was used to check for PCA group significant separation. Then a PLS-DA (2CV validation and permutation test) was performed and the VIP (metabolites responsible for the separation between the two groups) were obtained. Initially, features with VIP scores

higher than 1.5 were retained. Kruskal-Wallis test was applied to identify significant differences of the selected feature (VIP) expression between the groups (parasitized and non parasitized). Pertinence of selected features was also checked in the LC-MS chromatograms, and finally features with VIP scores higher than 2 were retained.

2.4.4. Peptide *de novo* sequencing

Peptide *de novo* sequencing, which consists in the determination of peptide sequences directly from tandem mass spectrometry (MS/MS) data, was carried out with the PEAKS Studio 7.5 (Bioinformatics Solutions, Waterloo, Ontario, Canada) software. PEAKS outputs a peptide significance score (-10lgP) (Ma et al. 2003; 2005). Error tolerances for *de novo* sequencing were set as follows: precursor mass 15 ppm and fragment ion 0.5 Da. PEAKS software can also identify amino-acid post-translational modifications, and modified peptides are evaluated with the same score (-10lgP) (Zhang et al. 2012). After obtention of the peptide sequences they were matched to the Swissprot database without taxonomic restriction and coverage percentage (%) of the matched proteins were obtained.

2.5. Comparison of gill mucus metabolome and microbiome (Relationship between the gill mucus metabolome and microbiome)

In order to study whether there was a significant relationship between the metabolites' VIP (> 1.5) and the OTUs' VIP (> 1.5), a Mantel test using Euclidean distances was performed. Relationship between the three haemoglobin metabolites overexpressed in *C. lunulatus* and the bacteria overexpressed in *C. lunulatus* (strictly anaerobic bacteria, Fusobacteria, sulphate-reducing bacteria and *Vibrio*) were analysed by Pearson correlation.

3. Results

3.1. *In vivo* test

Number of parasites per fish decreased significantly after an injection of *C. lunulatus* gill mucus to the three butterflyfish species the experiment was conducted on (*C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) (Figure 1). Parasite intensity decreased $62 \pm 17\%$ in treated *C. ornatissimus*, $70 \pm 6\%$ in treated *C. reticulatus*, and $55 \pm 7\%$ in treated *C. vagabundus*.

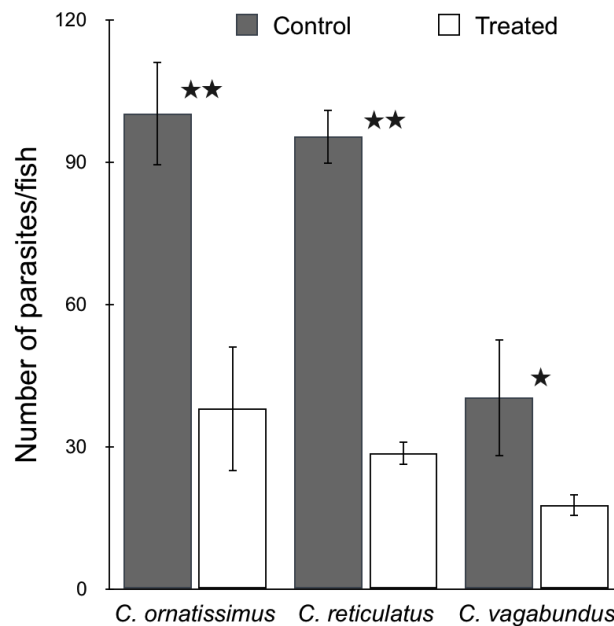


Figure 1. *In vivo* test results on *C. ornatissimus*, *C. reticulatus* and *C. vagabundus*. p-value < 0.05, p-value < 0.001.

3.2. Microbiome results

Microbiome of one non-parasitized butterflyfish species (*C. lunulatus*) and three highly parasitized butterflyfish species (*C. ornatissimus*, *C. reticulatus* and *C. vagabundus*) were studied. PCA analysis was used to assess the differences on the gill mucus microbiome between the parasitized and non-parasitized fish. The PERMANOVA test on the three principal components (PC1, PC2 and PC3) indicated that the groups were significantly different (p-value = 0.028) (Figure 2a). The PLS-DA model was accurate to predict bacterial differences (family level) between parasitized and non-parasitized fish (NMC = 0.16, p-value = 0.023) (Figure 2b). Twenty-six bacterial families presented a VIP score higher than 1.5. However, only 14 of these families were significantly more abundant in *C. lunulatus*

(Kruskal-Wallis, $p < 0.05$) (Figure 2c). Fusobacteriaceae was the bacterial family with the second highest VIP score, but presented the most significant difference between parasitized and non-parasitized fish (Figure 4). Spirochaetes (Spirochaetaceae and Sphaerochaetaceae) and Vibrionaceae were the other bacterial families that were significantly more abundant in *C. lunulatus*, representing more than 1% of total bacterial sequences (Figure 4).

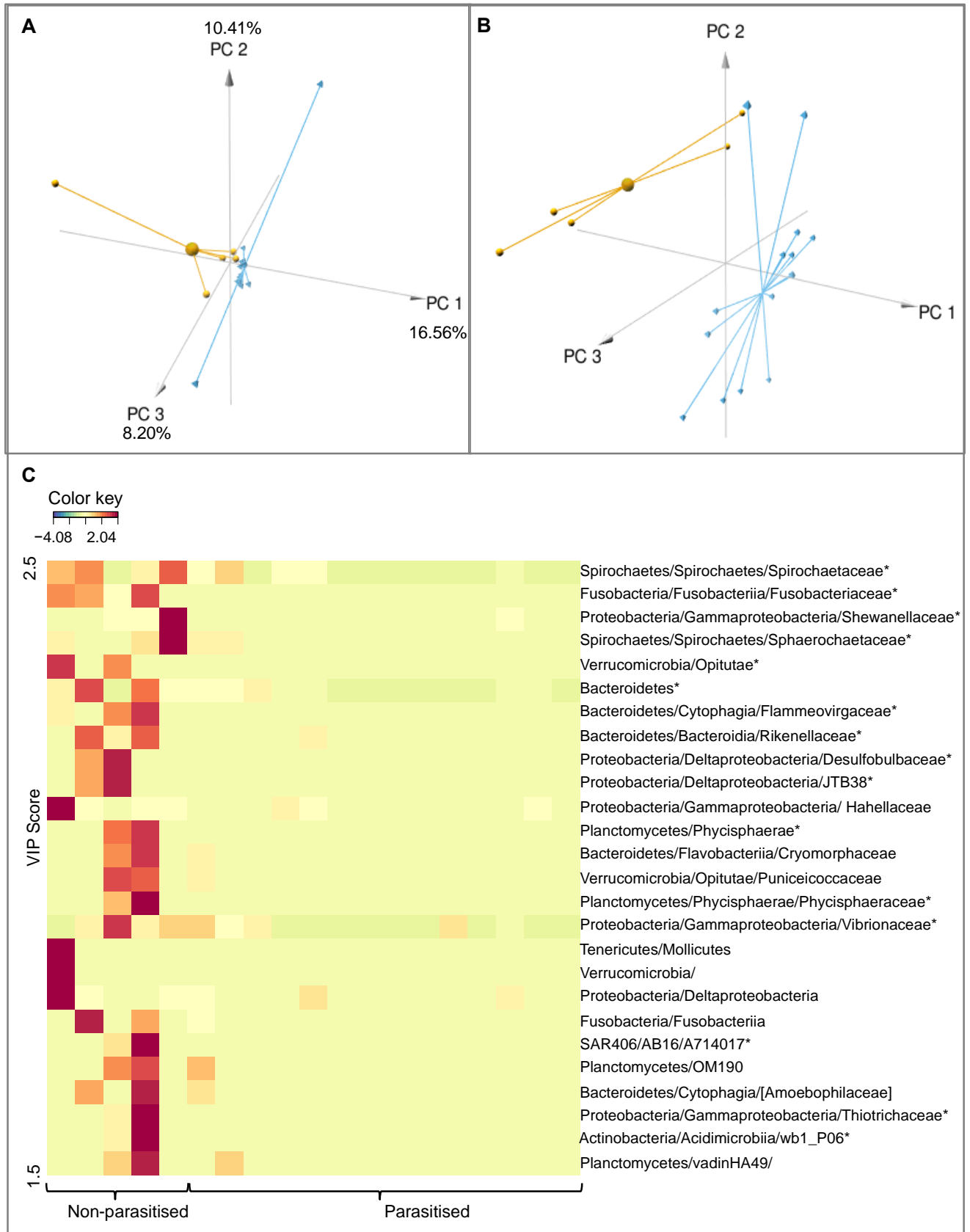


Figure 2. PCA (A) and PLS-DA (B) of the parasitized vs. non-parasitized fish and the heatmap (C) of the OTUs with VIP scores ordered from 1.5 to 2.5 (bottom to top). * indicates p-value < 0.05 (Kruskal-Wallis).

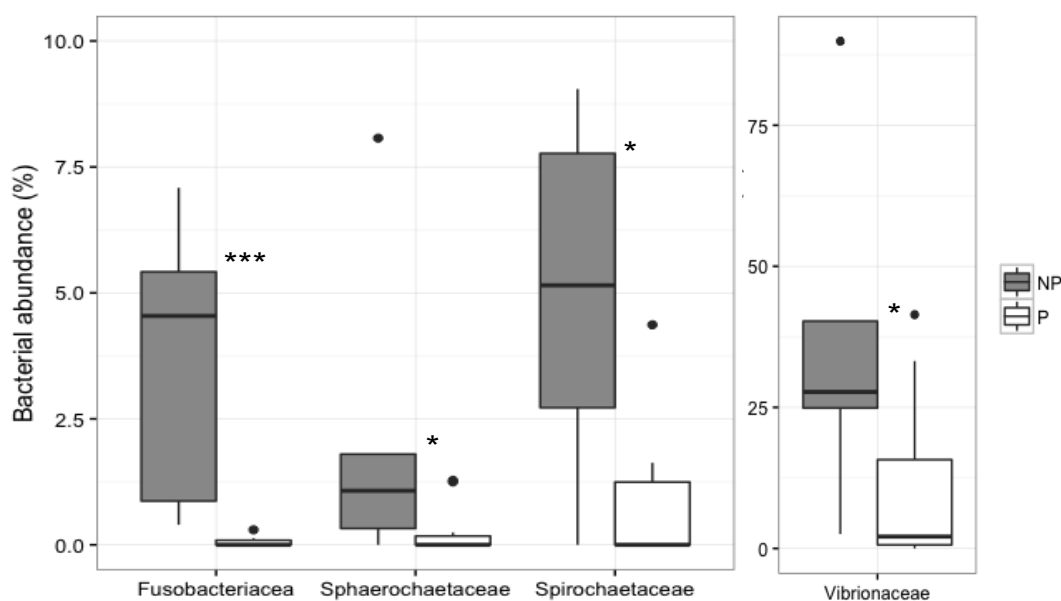


Figure 3. Relative bacterial abundances of VIP bacterial families found at > 1% in *C. lunulatus*. * p-value < 0.05; *** p-value < 0.001.

3.3. Metabolomic results

3.3.1. Multivariate analysis and VIP obtention

Metabolomics analyses were performed on both polar and apolar fractions. None of the VIP features obtained from the apolar fraction was significantly overexpressed in the non-parasitized fish (Supplementary material). Therefore, all the following results, including VIP obtention and identification are based on the analysis of the polar fraction.

Principal component analysis (PCA) was used to assess the metabolic differences between the sample groups (parasitized vs. non-parasitized). The PERMANOVA test on the three principal components (PC1, PC2 and PC3) indicated that the groups in the polar fraction were significantly different (p-value < 0.001) (Figure 4a). The PLS-DA model was accurate to predict metabolite differences between parasitized and not parasitized fish (NMC = 0.077, p-value = 0.001) (Figure 4b). 190 over 1835 identified features after feature alignment presented a VIP score higher than 1.5 and they were all significantly overexpressed in the non-parasitized fish (*C. lunulatus*) (Figure 5). Features with VIP scores > 1.5 were checked in the LC-MS chromatograms and due to signal intensity only VIP > 2 (43 features) were retained for further VIP characterisation from HR-LCMS analysis (Figure 4c). All the 43

metabolites with VIP scores higher than 2 presented concentrations significantly higher in the non-parasitized fish than parasitized fish (Kruskal-Wallis p-value > 0.001).

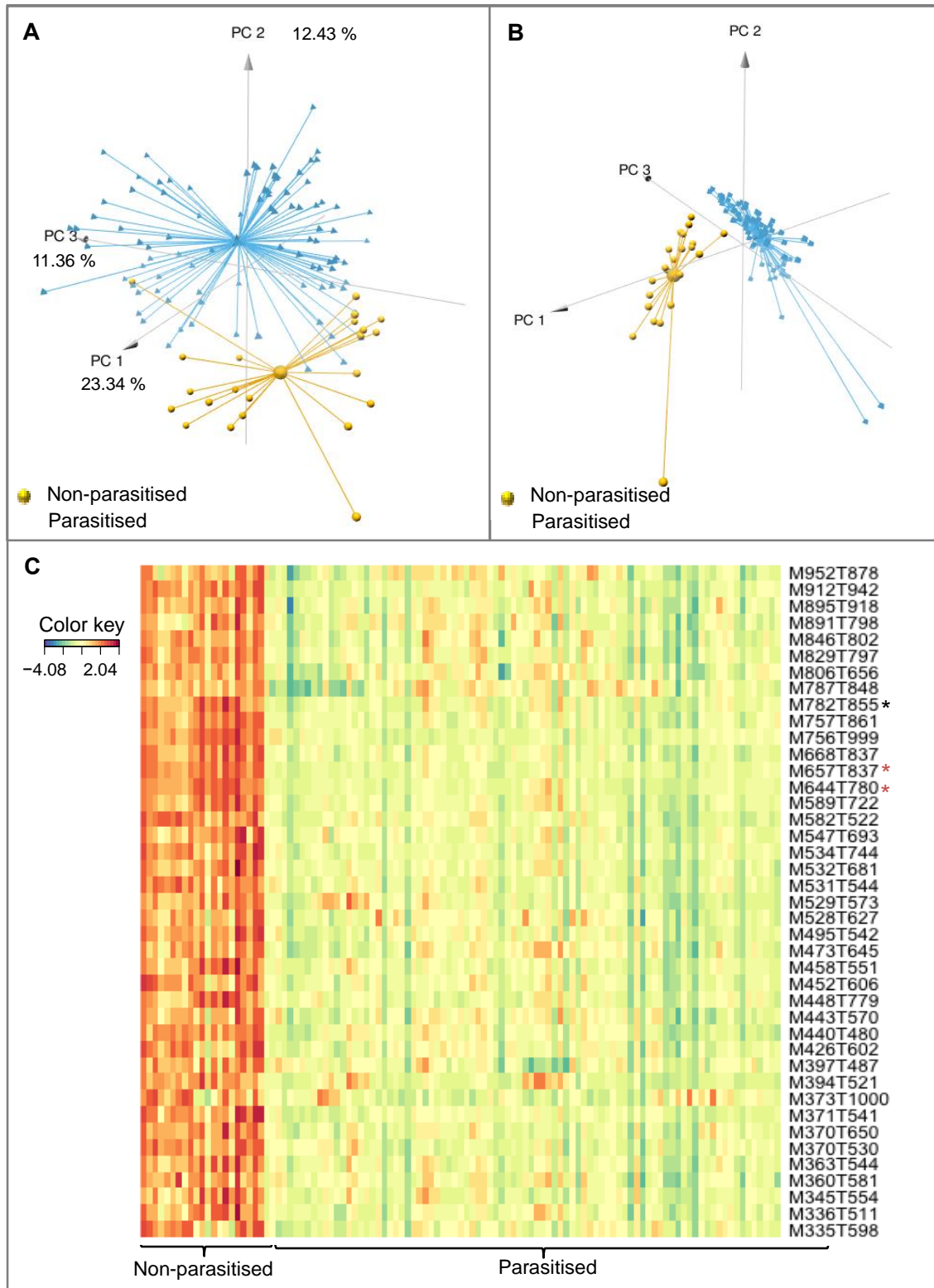


Figure 4. PCA (A) and PLS-DA (B) of the parasitized vs. non-parasitized fish and the heatmap (C) of the metabolites with VIP scores higher than 2 (polar fraction). * indicate peptides that were enriched in the acidic extraction. * indicate peptides whose approximate sequence was obtained by the PEAKS software.

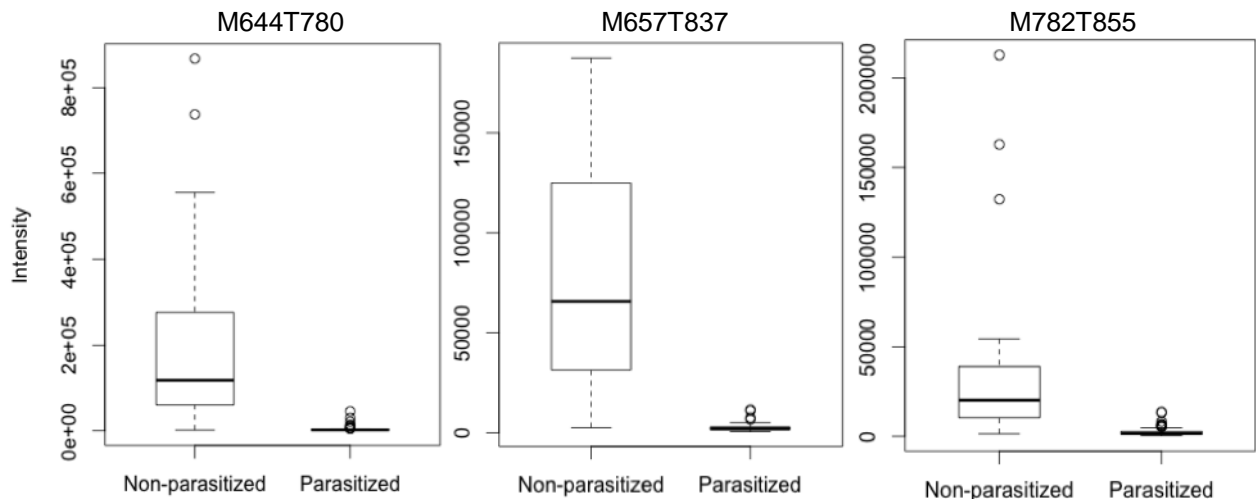


Figure 5. Boxplot of three peptides over-expressed in *C. lunulatus*.

3.3.2. VIP characterisation

Four different *C. lunulatus* extracts (polar fractions) were analysed by UHPLC-HRMS/MS in order to obtain a higher mass accuracy of the 43 features with VIP scores higher than 2. Forty-two of the features were multicharged ions corresponding to metabolites with masses ranging from 720 Da to 4556 Da. 17% of the metabolites presented a mass <1000 Da, 48% of the metabolites presented a mass between 1000-2000 Da and 33% of the metabolites presented a mass > 2000 Da (Figure 3a). LC-MS/MS profiles of some of the most important VIPs were characteristic of peptides, with several multi-charged ions and typical fragmentations (Figure 3b). Therefore, we decided to optimise the extraction procedure using an acidic extraction to selectively precipitate large proteins while enhancing the solubility of peptides. The UHPLC-HRMS/MS analysis of the acidic extract showed enrichment in three VIPs > 2 (M644T680, M657T837 and M782T855) (Figure 4). An acidic extraction of one heavily parasitized species (*C. reticulatus*) was also performed and we did not find any of the VIPs previously identified. The *de novo* sequencing done with the PEAKS software and the Swissprot database was achieved for the two most abundant VIP peptides at m/z 644.8725 and m/z 657.3557, which seem to match to amino acids sequences (respectively 10 and 30 residues) from the β -chain of the haemoglobin of the Japanese amberjack *Seriola quinqueradiata* (Table 1).

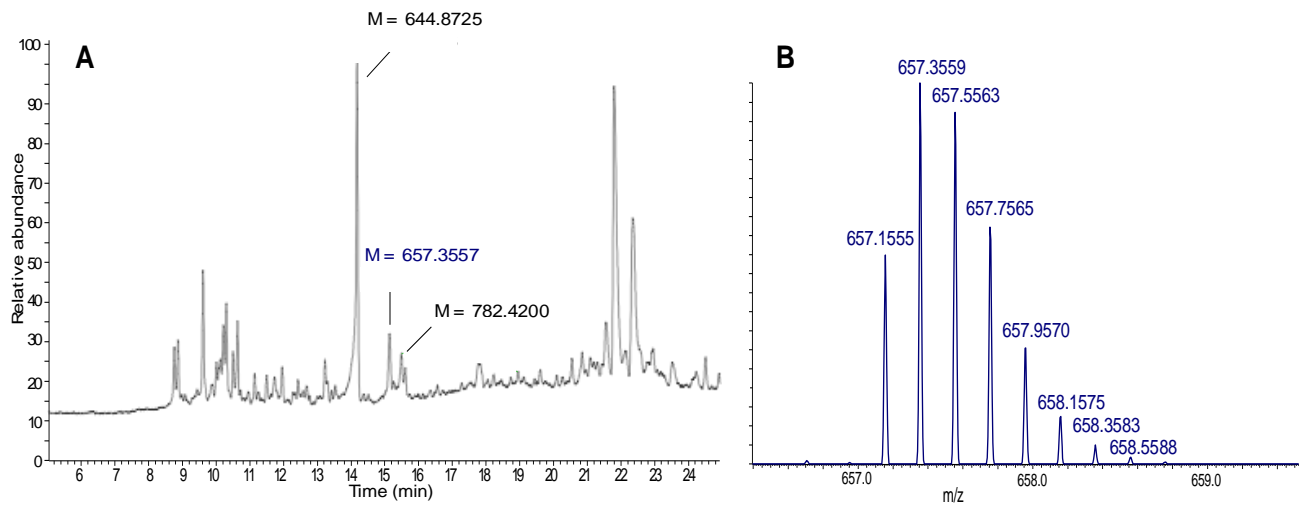


Figure 5. a) UHPLC/MS chromatogram of a *C. lunulatus* polar extract. Peaks detailed with mass (M in Da) and retention time (RT in min.) are three VIP peptides. b) Multi-charged isotopic cluster of the peptide with RT = 15.19 min.

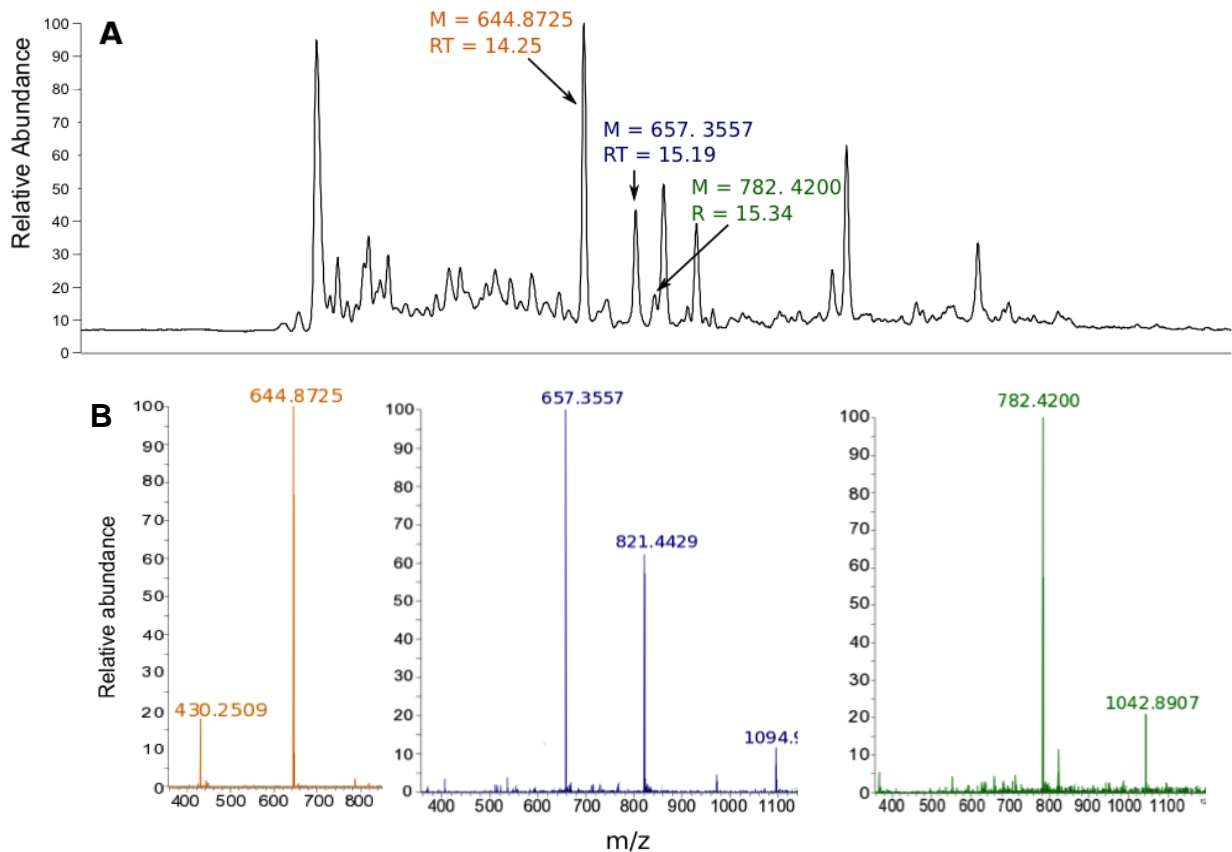


Figure 6. UHPLC (A) chromatogram of a *C. lunulatus* acidic extract. Peaks with detailed mass (M, Da) and retention time (RT, min) are three VIP peptides enriched in the acidic extraction. (B) Mass spectra of the three VIP peptides showing several multi-charged ions.

3.4. Relationship between metabolome and microbiome

The Mantel test revealed a strong correlation ($R = 0.62$, $p\text{-value} = 0.001$) between the metabolites and OTUs responsible for the differences between parasitized and non-parasitized fish ($VIP > 1.5$). Pearson correlation between the overexpressed bacteria in *C. lunulatus* and the three haemoglobin-derived peptides (M644T780, M657T837 and M782T855), showed that peptide M657T837 displayed the strongest correlation with Fusobacteriaceae ($\rho = 0.85$), while M644T780 and M782T855 displayed strong correlations with Spirochaetaceae ($\rho_{M644T780} = 0.79$ and $\rho_{M782T855} = 0.81$ respectively) and Sphaerochaetaceae ($\rho_{M644T780} = 0.91$ and $\rho_{M82T855} = 0.91$ respectively). The three hemoglobin-derived peptides had the weakest correlations with Vibrionoaceae (Figure 7).

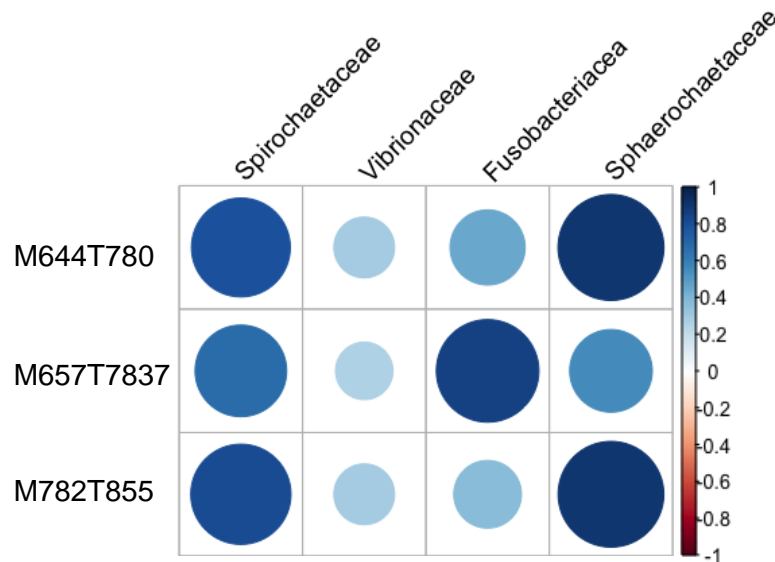


Figure 7. Pearson correlation between the three haemoglobin-derived peptides overexpressed in *C. Lunulatus* and bacteria overexpressed in *C. lunulatus*. Relative circle size indicates magnitude of correlation.

4. Discussion

Most butterflyfishes are parasitized by gill monogenean parasites. To date, only one butterflyfish species, *C. lunulatus*, has never been found parasitized by gill monogeneans (Reverter et al. 2016). *C. lunulatus* lives among parasitized butterflyfishes, and its similar ecology, behaviour and phylogenetic position cannot explain this marked absence of gill monogeneans. In this study, we have analysed the role of *C. lunulatus* gill mucus in the lack

of gill monogeneans in this species. We found that *C. lunulatus* mucus reduced significantly the number of parasites in three highly parasitized species, indicating a possible antiparasitic activity of *C. lunulatus* gill mucus. In our experiment, control fish followed the exact same stress as treated fish, but no mucus or other substance was injected on their gills. Therefore, we cannot exclude that the decrease in the number of parasites could be related to fish innate immune response to exogenous substances (gill mucus of another fish species), or even related to a decrease in oxygen diffusion due to an increase of mucus quantity.

Host commensal microbiota influences host physiology, playing an active role regulating host immune homeostasis (Wu and Wu, 2012). Fish microbiota homeostasis is important to avoid proliferation of pathogenic bacteria, and recent studies have shown that fish skin and gut isolates displayed antibacterial and antifungal properties against fish and human pathogens (Sanchez et al. 2012; Boutin et al. 2013, 2014; Lowrey et al. 2015). In a previous study we characterised the core microbiota of three highly parasitized butterflyfishes and the non-parasitized *C. lunulatus* (Chapter 5). In this study we found that the strictly anaerobic bacteria Fusobacteria and Spirochaetes and Vibrionaceae (facultative anaerobic) were significantly more abundant in *C. lunulatus*. These results indicate that *C. lunulatus* gill mucus might have less oxygen concentrations than the three highly parasitized butterflyfishes. Fusobacteria produce a short-chain fatty acid, butyrate, that provides benefits to mammal hosts, enhancing mucus production and acting as an anti-carcinogen and anti-inflammatory (von Engelhardt et al. 1998; Andoh et al. 1999). Although, Fusobacteria role in fish is not well established, we can hypothesise that if they do enhance mucus production, this would increase gill mucus layer thickness, increasing oxygen diffusion distance, and eventually promoting higher levels of hypoxia (Hess et al. 2015). Spirochaetes bacteria are associated with a wide range of human and animal pathologies such as Lyme disease, syphilis, and dermatitis (Radolf et al. 1995; 2012; Fraser et al. 1998). Spirochaetes have also been found associated to marine invertebrates such as sponges and molluscs, some of them being infectious, however to date, we know little of their presence and role in fish (Neulinger et al. 2010). Vibrionaceae bacteria are often reported as opportunistic pathogens causing numerous diseases on a wide range of marine organisms including fish, shellfish and corals (Diggles et al. 2000; Sung et al. 2001; Cervino et al. 2008). Increased mucus production on stressed or infected fish is well reported, therefore a higher abundance of Vibrionaceae bacteria and

eventually Spirochaetes could also be related to an increase in mucus production and decrease of oxygen (Ferguson et al. 1992; Hess et al. 2015).

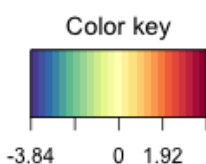
We have also studied the overexpressed metabolites in *C. Lunulatus* and we identified several peptides that could be derived the β -subunit of haemoglobin that were absent in other fish species, which were especially well correlated to Fusobacteria and Spirochaetes. Haemoglobin (Hb) is an iron-containing oxygen-transport metalloprotein with a quaternary structure composed of two α -globin and two β -globin chains (Anthea et al. 1993). Hb is mainly found in the red blood cells of vertebrates, however, it is also expressed in nonerythroid cells such as alveolar epithelial cells, macrophages, retinal pigment epithelial cells and hepatocytes (Saha et al. 2014). Haemoglobin play a crucial role in the transport of oxygen to the cells, and several studies reported an increased expression of haemoglobin under low oxygen levels in organisms such as fish, plants or humans (Hunt et al. 2002; Heinicke et al. 2003). Haemoglobin up-regulation has also been observed in several fish and shellfish tissues such as skin, gill or internal organs following *Vibrio* infections (Bao et al. 2013; Meloni et al. 2015). In addition to its primordial function as an oxygen carrier, haemoglobin is a known source of biologically active peptides that exhibit varied functions such as analgesic, antimicrobial and antiparasitic (Takagi et al. 1979; Liepke et al. 2003; Ullal and Noga, 2010). Ullal et al. (2008) found that expression of three β -chain haemoglobin peptides (Hb β P-1, Hb β P-2 and Hb β P-3) increased after ich (*Ichthyophthirius multifiliis*) infection in gill epithelium of channel catfish (*Ictalurus punctatus*). Hb β P-1 displayed a moderate activity against bacterial pathogens, but had a high specific activity against different ectoparasite species (Ullal et al. 2010). Cell-free Hb is toxic to the organism because it generates reactive oxygen species (ROS) that perturb the innate immune homeostasis (Lee and Ding, 2013). However, Jiang et al. (2007) found that the Hb redox activity is specifically activated by microbial virulence factors such as lipopolysaccharides (LPS) and proteases. A recent study showed that Hb is specifically and progressively cleaved by the extracellular proteases of the invading microbe (Du et al. 2010). Some active Hb peptides then display a dual function, they bind to the pathogen-associated molecular patterns (PAMPs) and they produce cytotoxic free radicals, killing the pathogen in proximity and limiting at the same time diffusion of free radicals in the host (Du et al. 2010). Altogether, these results indicate that the hemoglobin peptides found in *C. lunulatus* might have biological activities such as antiparasitic and thus be responsible for the absence of monogeneans in this fish species. We

suggest that expression of hemoglobin peptides in *C. lunulatus* might be enhanced either from a low oxygen environment, or after microbial cleaving of the hemoglobin protein. In turn, some of these peptides, such as peptide M657T837, which possess a high homology to Hb β P-3, might display antimicrobial and anti-parasitic activities. Peptide purification, further peptide sequence confirmation of purified peptides, peptide synthesis and evaluation of the biological activities are being currently performed.

It is noteworthy to raise the question whether a durable association between potentially pathogenic *Vibrio* bacteria and *C. Lunulatus* could exist, or the observed higher *Vibrio* abundance in *C. lunulatus* was punctual. *C. lunulatus* has been observed to feed preferentially on bacterial mats of coral disease lesions, harbouring high densities of *Vibrio* bacteria (Arotsker et al. 2009; Chong-Seung et al. 2011). Such foraging habitats could promote *Vibrio* transfer from diseased corals to *C. lunulatus* external organs such as gills. However, durability and physiological consequences of associations between *Vibrio* and *C. Lunulatus* have yet to be properly studied.

In summary, we have studied the gill mucus of *C. lunulatus* in comparison to other parasitized butterflyfish species to investigate the potential role of *C. lunulatus* mucus in the absence of monogeneans. *C. lunulatus* gill mucus induced a decrease of the number of parasites in three highly parasitized species. We further studied the microbiome and metabolome of *C. lunulatus* gill mucus and found a higher presence of Fusobacteria, Spirochaetes and Vibrionaceae bacteria that was positively correlated with an overexpression of hemoglobin peptides. These results suggest that bacteria might play a role enhancing production of hemoglobin peptides, either through the decrease of the oxygen levels or by cleaving the hemoglobin protein. Hemoglobin peptides present a wide array of bioactivities, therefore we hypothesise that they might play a role preventing monogenean infection in *C. lunulatus*. Further evaluation of purified peptides biological activities will be performed in the laboratory.

SUPPLEMENTARY INFORMATION



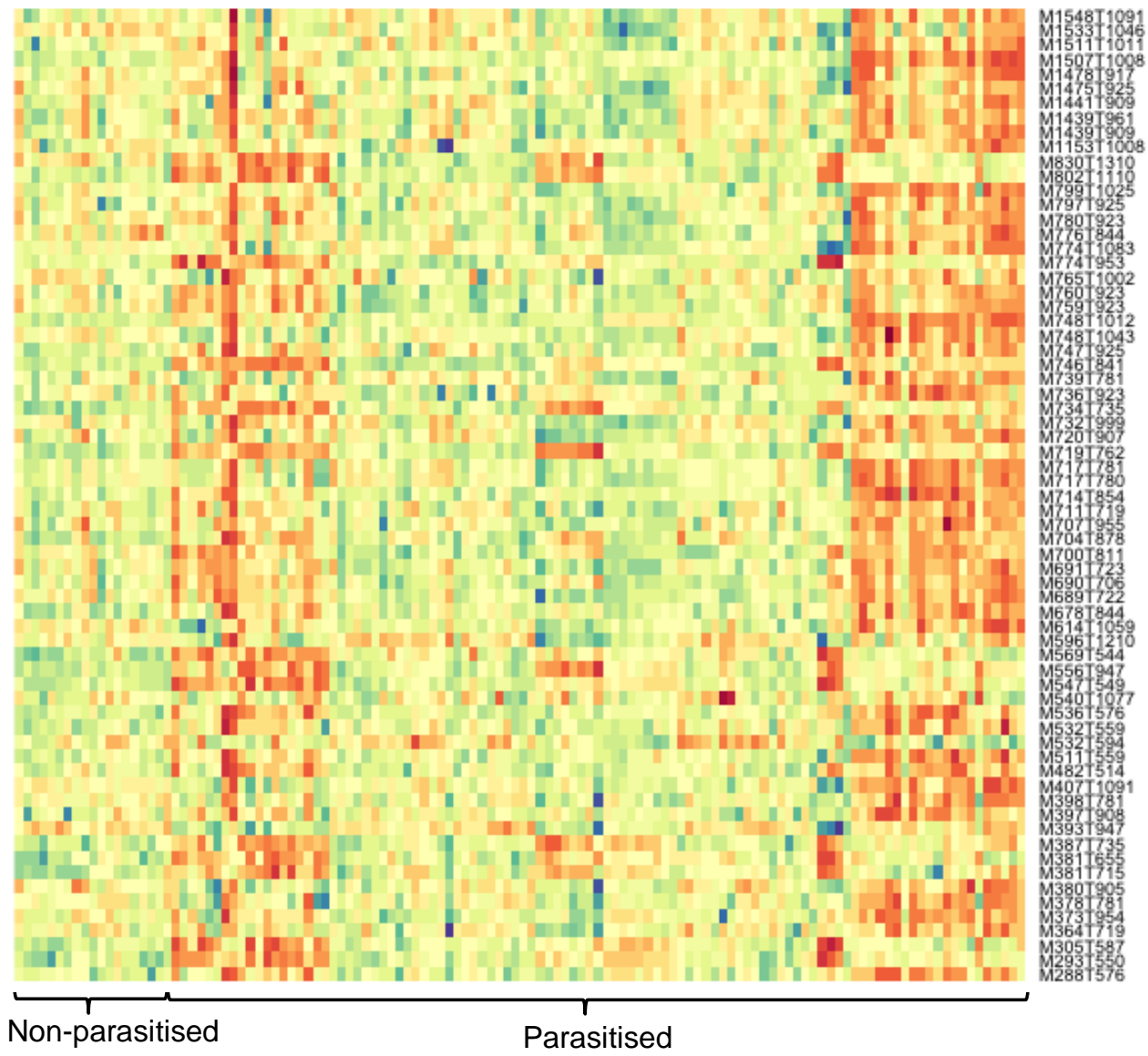


Figure S1. Heatmap from the apolar fraction of the metabolites with VIP scores higher than 2.

Part 2

Fish-parasite interactions in aquaculture:
Orbicular batfish – bacterial interactions



Scientific context and model species

Scientific context and model species

Scientific context

Fish and fishery products are an important source of proteins with crucial nutritional value, especially in developing and densely populated countries (FAO, 2014). Global fish production has been growing steadily with an average annual rate of 3.2% in the last five decades. Since fish captures have remained stable at about 90 million tones since the 1990s, aquaculture has been the main source for increasing fish supply. Global aquaculture production attained 90.4 million tones in 2012, and according to estimates, world food fish aquaculture production rose by 5.8% in 2013. World aquaculture production is imbalanced, with Asia accounting for about 88% of aquaculture production by volume, and China itself for nearly 62%. Oceania on the other side produced only 0.28% (184 191 tones) of world aquaculture production in 2012 (FAO, 2014).

French Polynesians, as other remote maritime areas, rely on sea resources as an important part of their daily diet, and are among the highest fish consuming nations in the world, with a fish consumption per capita of 54 kg/year (Dewailly et al. 2008a, 2008b). Although, local fishing is still the main source of fish and seafood in French Polynesia, local aquaculture has emerged during the last years.

Aquaculture in French Polynesia is dominated by the black pearl aquaculture (*Pinctada margaritifera cumingii*) that takes places mainly in the Tuamotu and Gambiers archipelagos. However, since the 1970s, there has been a local effort to develop other aquaculture sectors like the shrimp (*Litopenaeus stylirostris*) and the giant clam (*Tridachna maxima*) aquaculture. Fish lagoon aquaculture in Tahiti has been developed very recently with the main objective of promoting local sustainable farming to obtain high quality fish meat free of ciguatoxins and other ciguatera associated toxins (SPC, 2008). In 2001, the Fisheries Department of French Polynesia (DRMM) began aquaculture trials on three different marine finfish species: moi (sixfinger threadfin, *Polydactylus sexfilis*), pa'aihere manini (golden trevally, *Gnathanodon speciosus*), and paraha peue (orbicular batfish, *Platax orbicularis*). In 2006, the DRMM and the French Research Institute for the Exploitation of the Sea (Ifremer) targeted the orbicular

batfish (*Platax orbicularis*) as the priority candidate in lagoon aquaculture development in French Polynesia, due to its culture performance and its economic value (David et al. 2010). The orbicular batfish is indeed a highly appreciated fish by the local Polynesian and Chinese communities for its meat quality and taste, that has become uncommon in French Polynesian waters due to overexploitation. In 2010, a national hatchery was built, which has nowadays the total control of the orbicular batfish biological cycle, and in 2011 the first private orbicular batfish farm was established. In 2014, 13 tonnes of *P. orbicularis* were produced in Tahiti accounting for nearly 200.000 USD (FAO Global Aquaculture Production Database).

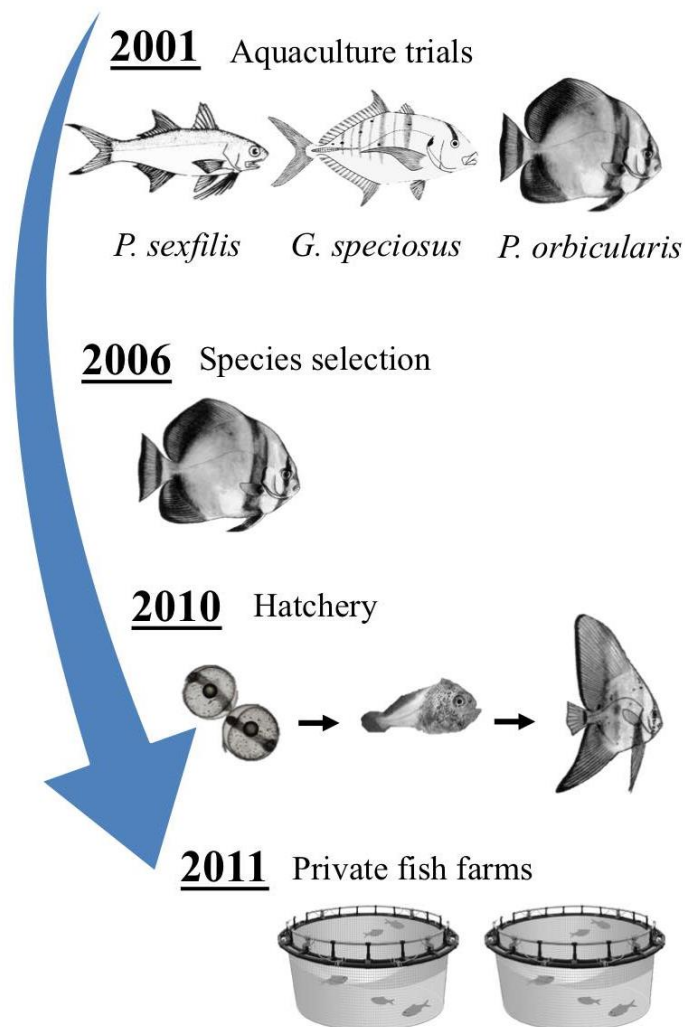


Figure 1. *P. orbicularis* aquaculture development in Tahiti.

Platax orbicularis

Platax orbicularis (Forsskal, 1775; Ephippidae) is a tropical batfish found throughout the Indo-Pacific, which is normally associated to coastal ecosystems such as coral reefs and mangroves (Barros et al. 2015). It reaches a maximum size of 40 cm and is usually found at depths of 30 m. *Platax* juveniles present extremely tall and deep dorsal and anal fins, which proportionally decrease in size with growth, as the adults become more "bat-like" with round and deeply compressed bodies (Cavalluzzi et al. 2000). The juveniles are usually found in shallow water, drifting around floating materials and mimicking dead leaves on the surface of the water through their coloration, body shape and swimming behavior (Barros et al. 2008). *Platax* fingerlings feed on algae during the day and zooplankton during the night (Barros et al. 2008). Although ecology of adult orbicular batfish is not well studied, they are described as omnivorous fish (Leis et al. 2013).

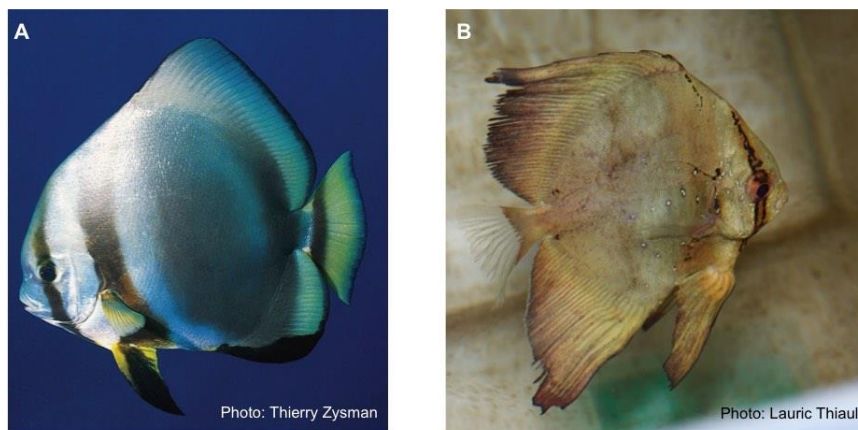


Figure 2. Adult (A) and fingerling (B) of orbicular batfish (*P. orbicularis*).

Orbicular batfish are also produced in Taiwan for food purposes and in Thailand as ornamental fish for the aquarium market. In Tahiti, *P. orbicularis* fingerlings are produced in the indoor bio-secured Vaia hatchery and then transferred and reared in floating offshore net-cages in the lagoon of Tahiti Iti. However, *P. orbicularis* fingerlings have been suffering severe mortality episodes shortly after the transfer due to bacterial disease caused jointly by *Tenacibaculum maritimum* and *Vibrio harveyi*. Around five days after the transfer, fish show signs of disorientation, lost of appetite and reduced swimming ability. Shortly after, fish start showing symptoms of disease with white patches on tegument that evolve in necrosis,

damaged pectoral and caudal fins and in some cases ulcers in the mouth too (Saulnier et al. pers. comm).

Tenacibaculum maritimum (Flavobacteriaceae) is a Gram-negative, filamentous bacteria formerly known as *Flexibacter maritimus* that causes the marine disease tenacibaculosis, which affects a large number of fish species and causes heavy aquaculture losses worldwide (Avendano-Herrera et al. 2006b). Several authors studied the infection pathway of *T. maritimum*, and although it isn't completely clear, it seems that *T. maritimum* would attack the body surfaces of immunosuppressed or already abraded fish, causing external lesions (ulcers, necrosis, eroded mouths, frayed fins and tail-rot), and once it penetrates the body, it would easily spread along internal organs causing septicemia (Avendano-Herrera et al. 2004, 2006b; Failde et al. 2013). An increased prevalence and severity of the disease has been reported at higher temperatures (above 15°C) and salinities (30 to 35‰) as well as with low water quality (Avendano-Herrera et al. 2006b).

Besides, external lesions caused by tenacibaculosis could then favor the entrance of other pathogenic bacteria such as *Vibrio sp.* (Avendano-Herrera et al. 2004). *Vibrio harveyi* (Vibrionaceae) is a Gram-negative, bioluminescent bacteria that is found normally in the intestinal microbiota of aquatic vertebrates and invertebrates. Although, it is often considered to be an opportunistic pathogen, *V. harveyi* has also been reported to be a primary disease-causing agent with highly virulent strains (Austin and Zhang, 2006). Several diseases such as gastro-enteritis, skin ulcers and dermal and eye lesions as well as mortality episodes in different fish species have been attributed to *V. harveyi* infections (Bertone et al. 1996; Ishimaru and Muroga 1997; Lee et al. 2002).

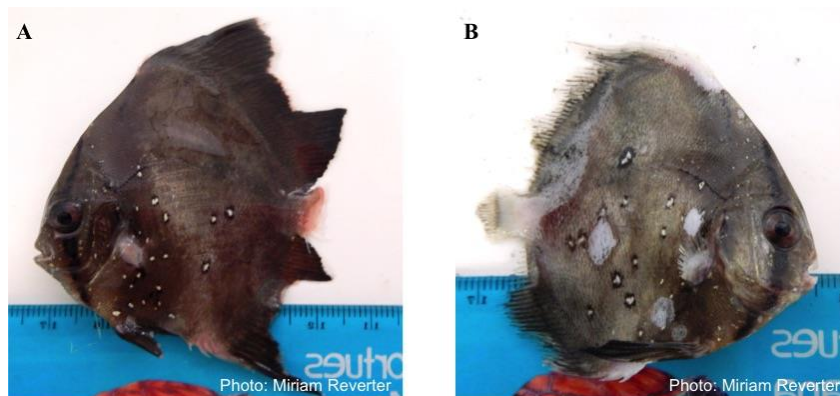


Figure 3. *P. orbicularis* fingerlings with tenacibaculosis at different infectious stages.

The current treatment for tenacibaculosis and vibriosis is done by chemotherapy and antibiotic administration. Several studies have shown that bath treatments might be more effective against tenacibaculosis than oral treatments, since infected fish become anorexic immediately post-infection (Soltani et al. 1995). An alternative treatment to antibiotics is the use of immersion baths with surface acting disinfectants such as formalin or hydrogen peroxide. However, these baths are often aggressive to fish and some physiology problems have been noted (McVitar and White, 1979; Avendano-Herrera et al. 2006a, 2006b).

This chapter aims to study alternative more sustainable solutions for the prevention and treatment of the marine tenacibaculosis in *P. orbicularis*, in order to decrease the severe mortality episodes currently encountered in Tahiti. Since *P. orbicularis* aquaculture in Tahiti takes place close to the coral reef, synthetic persistent molecules should be restricted. I investigated the potential of several local Polynesian plants (garlic, turmeric, noni, ginger and tamanu) and algae (*Asparagopsis taxiformis*, *Padina boryana*, *Turbinaria ornata*, *Dichotoma marginata*, *Lingbya majuscula* and *Anabaena torulosa*) against the two major pathogenic agents (*Vibrio harveyi* and *Tenacibaculum maritimum*), by evaluating plant and algae antibacterial activities, studying the capacity of plants and algae to increase level of expression of two immune-related genes in orbicular batfish (*Platax orbicularis*) and finally testing *in vivo* efficacy of orally administered plants and algae in reducing infected (*Tenacibaculum maritimum*) orbicular batfish mortality.



CHAPTER VII

Use of plant extracts as an alternative to chemotherapy: Current status and future perspectives

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., Sasal, P. (2014). Use of plant extracts as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture* 433, 50-61. (IF = 1.8)

Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives.

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Abstract

Aquaculture is the main source to increase fish supply. Fast development of aquaculture and increasing fish demand leads to intensification of fish culture, magnifying stressors for fish and thus heightening the risk of disease. Until now, chemotherapy has been widely used to prevent and treat disease outbreaks, although use of chemical drugs has multiple negative impacts on environment and human health e.g. resistant bacterial strains and residual accumulation in tissue. Hence, disease management in aquaculture should concentrate on environmental friendly and lasting methods. Recently, increasing attention is being paid to the use of plant products for disease control in aquaculture as an alternative to chemical treatments. Plant products have been reported to stimulate appetite and promote weight gain, to act as immunostimulant and to have antibacterial and anti-parasitic (virus, protozoans, monogeneans) properties in fish and shellfish aquaculture due to active molecules such as alkaloids, terpenoids, saponins and flavonoids. However, as it is a relatively emerging practice there is still little knowledge on the long-term effects of plant extracts on fish physiology as well as a lack of homogenization in the extract preparation and fish administration of the plant extracts. This article aims to review the studies carried out on the use of plant products on fish aquaculture and their biological effects on fish such as growth promoter, immunostimulant, antibacterial and anti-parasitic. It also intends to evaluate the current state of the art, the methods used and the problems encountered to their application to the aquaculture industry.

Keywords: plant extracts, fish aquaculture, disease resistance, immunostimulant, natural products.

Highlights

- Diseases outbreaks are becoming more recurrent and conventional treatment methods have multiple environmental and health disadvantages.
- There is an increasing use of natural products in aquaculture for disease control due to their multiple bioactivities.
- There is a great need to normalize the use of natural products in aquaculture in order to assure their efficacy.
- Algae natural products should be favoured to avoid any exogenous molecule to be introduced in the sea.

1. Introduction

Fish and fishery products represent a very valuable source of protein and essential micronutrients for balanced nutrition and good health. In 2009, fish accounted for 17% of the world population intake of animal protein and 6.5% of all protein consumed. World fish food supply has grown considerably in the last five decades, with an average growth rate of 3% per year in the period 1961-2009. Since fish capture has remained stable at about 90 million tonnes since 1990, aquaculture has been revealed as the main source for increasing fish supply. World aquaculture production reached 62 million tonnes in 2011 (excluding seaweeds and non-food products), with an estimated value of US\$ 130 billion. Aquaculture is the fastest-growing animal food producing sector with an average annual increase of 6% per year in the period 1990-2010 (FAO, 2012).

However, world aquaculture production is vulnerable and an increase of diseases outbreaks has been reported due to culture intensification, resulting in partial or total loss of production (Bondad-Reantaso et al., 2005). Factors such as overcrowding, periodic handling, high or sudden changes in temperature, poor water quality and poor nutritional status contribute to physiological changes in fish such as stress or immunosuppression and thus, heighten susceptibility to infection. Moreover, high concentrations of fish and lack of sanitary barriers facilitate the spread of pathogens, producing high mortality levels (Naylor et al., 2000; Cabello, 2006; Quesada et al., 2013).

In order to avoid economic losses related to sanitary shortcomings, several veterinary drugs are commonly used in aquaculture to prevent or treat diseases outbreaks. Antimicrobials and other veterinary drugs are administered regularly as additives in fish food or sometimes in baths and injections and are used as prophylactics (prevent diseases before they occur), therapeutics (treat sick animals) or growth promoters (Rico et al., 2013).

Nevertheless, the use of veterinary drugs is becoming more restricted since they present numerous side-effects for the environment and health safety. For example, massive use of antibiotics have resulted in the development of resistant bacteria strains (e.g. Mirand and Zemelman, 2002 ; Seyfried et al., 2010) or the presence of residual antibiotics in the muscle of commercialized fish and thus has potential consequences on human health (e.g. Cabello et al., 2006; Romero Ormázabal et al., 2012). The use of drugs like trichlorfon or praziquantel in bath treatments for ectoparasites has also numerous disadvantages like development of resistance (Umeda et al., 2006), hazardous for animal health (Kiemer and Black, 1997; Forwood et al., 2013) and environmental disadvantages. Vaccination has also been regarded as a potential treatment against diseases outbreaks in aquaculture. However, commercial vaccines are too expensive for a widespread use by fish producers and they have the downside that a single vaccine is effective against only one type of pathogens (Sakai, 1999; Pasnik et al., 2005; Harikrishnan et al., 2011a).

Considering the potential harm of veterinary drug treatments on environment and human health and in some cases their limited efficacy, disease management should concentrate on harmless, preventive and lasting methods. Moreover, disease outbreaks are frequently associated with fish fitness and health, most of pathogens being opportunistic and taking advantage of immunocompromised or stressed fish, thus alternate solutions should maximize fish immunity and fitness to avoid and face pathogen infections (e.g., Ruane et al., 1999; Davis et al., 2002; Iguchi et al., 2003; Ashley 2007). Some of the proposed solutions are the use of natural products (plant extracts) or probiotics (beneficial microbial strains) in the culture of fish and shrimps (e.g., Panigrahi and Azad, 2007; Makkar et al., 2007; Citarasu et al., 2010; Lee and Gao, 2012 ; Mohapatra et al., 2013). Finally, there is an increasing interest in consuming organic and environmentally friendly food. Thereafter, the limitation of chemical products in aquaculture and the use of natural treatments could enhance the consumption of aquaculture products.

In this article we aim to review the potential of plant extracts as a sustainable and effective substitute of chemical treatments in fish aquaculture. In order to be as exhaustive as possible we performed several databases crossed searches with the keywords « plant extracts » and « fish aquaculture » (Web of Knowledge, PubMed, Scopus, ScienceDirect, SpringerLink Journals and Wiley Online Library) and we found 115 results that matched our criteria. The results clearly show that there is an increasing number of published studies highlighting the potential application of natural products and plant extracts in aquaculture either as immunostimulant or to fight parasite diseases (Figure 1).

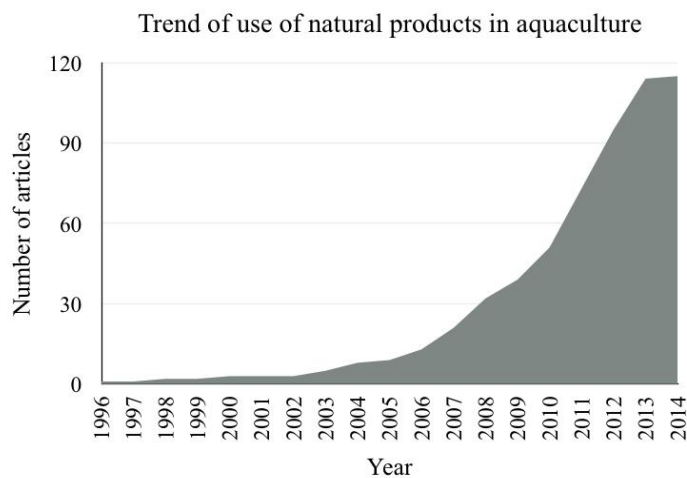


Figure 1. Number of published articles about the use of plant, algae or natural products in aquaculture.

2. Potential of plant extracts in aquaculture

Plant extracts have been reported to favor various activities like anti-stress, growth promotion, appetite stimulation, enhancement of tonicity and immunostimulation, maturation of culture species, aphrodisiac and anti pathogen properties in fish and shrimps aquaculture due to the active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or essential oils (Citarasu, 2010; Chakraborty and Hancz, 2011). Besides, their use could reduce costs of treatment and be more environmentally friendly as they tend to be more biodegradable than synthetic molecules and they are less likely to produce drug resistance on parasites due to the high diversity of plant extract molecules (Logambal et al, 2000; Blumenthal et al., 2000; Olusola et al., 2013).

2.1. Plant extracts as appetite stimulators and growth promoters

Several plant extracts are reported to stimulate appetite and promote weight gain when they are administered to cultured fish (Pavaraj et al., 2011; Harikrishnan et al., 2012a, Takaoka et al., 2011) (Table 1, 2). Shalaby et al. (2006) showed that food intake, specific growth rate and final weight of Nile tilapia (*Oreochromis niloticus*) increased when garlic was incorporated in the diet. In another study, grouper *Ephinephelus tauvina* fed with a diet supplemented with a mixture of methanolic herb extracts (Bermuda grass (*Cynodon dactylon*), Long pepper (*Piper longum*), stonebreaker (*Phyllanthus niruri*), coat buttons (*Tridax procumbens*) and ginger (*Zingiber officinalis*)) displayed 41% higher weight than fish fed with the control (Punitha et al., 2008). Ji et al. (2007a) showed that olive flounder (*Paralichthys olivaceus*) fed with a herbal mixture of medicated leaven (*Massa medicata fermentata*), hawthorne (*Crataegi fructus*), virgate wormwood (*Artemisia capillaries*) and *Cnidium officiale* (2:2:1:1) had higher weight gain than the control fish and showed higher total carcass unsaturated fatty acid content and lower carcass saturated fatty acid content, indicating that feeding with the herbal mixture improves fatty acid utilization. The authors suggested that this could be caused by a lower plasma triglyceride and high plasma HDL-CHO (high-density lipoprotein cholesterol) levels in the herbal mixture diets.

Besides, plant extracts have been shown to improve digestibility and availability of nutrients resulting in an increase in feed conversion and leading to a higher protein synthesis (Nya and Austin, 2009b; Citarasu, 2010; Talpur et al. 2013b). For example, Putra et al. (2013) showed that supplemented diet with 1% of ethanolic katuk extract (*Sauropus androgynous*) stimulated appetite, growth and improved food utilization (lower feed conversion ratio) in grouper *Ephinephelus coioides*. However, *E. coioides* fed with enriched diets with higher percentages of katuk extract (2.5 and 5%) presented lower growth. These results also indicate the importance of suitable dosing to obtain the desired effects and thus the need of further studies to chemically characterize extracts in order to be able to quantify active molecules and establish adequate doses.

2.2. Plant extracts as immunostimulants

The immune system is classified into innate (non-specific) and adaptive (specific) immune systems. The innate immune system is the first line of defense against invading pathogens and their major components are macrophages, monocytes, granulocytes, and humoral elements, including lysozyme or complement system (Harikrishnan et al., 2009b). An immunostimulant is a substance that enhances the defense mechanisms or immune response (both specific and non-specific), thus rendering the animal more resistant to diseases and external aggressions (Anderson, 1992).

An increasing interest in the use of plant extracts as fish immunostimulants has arisen in the last decade (Galina et al., 2009; Vaseeharan and Thaya, 2013). Several studies have monitored the immunological parameters after intraperitoneal injection or orally administered plant extracts on distinct fish species and they found that treated fish showed increased lysozyme activity, phagocytic activity, complement activity, increased respiratory burst activity and increased plasma protein (globulin and albumin) (e.g. Dügenci et al., 2003; Yuan et al., 2007; Wu et al., 2010) (Table 1, 2). Lysozyme plays an important role in the defense of fish by inducing antibacterial activity in the presence of complement (Harikrishnan et al., 2012b). Phagocytosis is one of the main mediators of innate immunity to pathogens, while respiratory burst is also a crucial effector mechanism for limiting the growth of fish pathogens (Divyagnaneswari et al., 2007). Increase in plasma protein, albumin and globulin, is considered as a strong innate response in fishes.

Other studies analyzed the hematological parameters which provides a clue of the fish health status and they found that erythrocytes, lymphocytes, monocytes, haemoglobin and haemocrit levels significantly increased in fish treated with plant extracts compared to control fish (e.g. Shalaby et al., 2006; Innocent et al., 2011; Harikrishnan et al., 2012a).

For example, Park and Choi (2012) showed that Nile tilapia (*Oreochromis niloticus*) fed with diets containing different doses of mistletoe (*Viscum album coloratum*) for a period of 80 days in which immunological parameters were monitored (lysozyme activity, respiratory burst activity, alternative complement activity and phagocytic activity), displayed an increase in activity. After that period, fish were challenged against *Aeromonas hydrophila* and treated fish had 42% more survivability than control group.

Another study showed that olive flounder (*Paralichthys olivaceus*) infected with the scutociliate *Philasterides dicentrarchi* presented less mortality (30 - 45%) when was fed with monkey head mushroom (*Hericium erinaceum*) enriched diet (0.1 and 1% respectively) compared to control group which had a mortality of 90%. Lysozyme activity and burst respiratory activity of olive flounder increased more in the treated groups, indicating that enhancement of immunological system leads to a better protection of olive flounder against *P. dicentrarchi* (Harikrishnan et al., 2011b).

Kim and Lee (2008) showed that non-specific immune response in juvenile olive flounder (*P. olivaceus*) fish was enhanced (increased phagocytes' respiratory burst, serum lysozyme and myeloperoxidase activities) with dietary supplementation of kelp (*Ecklonia cava*), most probably due to the high antioxidant and polyphenolic content in *E. cava*.

These results indicate that enriched diets with plant extracts have beneficial effects on fish health and enhance immune system and hence they could play an important role avoiding diseases outbreaks in aquaculture systems. However, in most cases mechanisms responsible for the physiological answer in fish are still unknown.

Plant	Fish	Type of extract	Type of administration	Lenght of treatment (days)	Growth promoter	Immunostimulant	References
<i>Allium sativum</i>	<i>Tilapia zillii</i>	Powder (bulb)	Oral	75	+		Jegade et al., 2012
<i>Cynodon dactylon</i>	<i>Onchorhynchus mykiss</i>	Powder	Oral	40	+	+	Oskooi et al., 2012
<i>Satureja khuzestanica</i>	<i>Cyprinus carpio</i>		Oral	35		+	Khansari et al., 2013
<i>Sauropus androgynus</i>	<i>Epinephelus coioides</i>	Ethanol (leaves)	Oral	70	+		Putra et al., 2013
<i>Urtica dioica</i>	<i>Onchorhynchus mykiss</i>	Water (leaves)	Oral	21		+	Dügenci et al., 2003
<i>Viscum album</i>	<i>Onchorhynchus mykiss</i>	Water (leaves)	Oral	21		+	Dügenci et al., 2003

Table 1. Growth-promoter and immunostimulant effect of plant extracts on fish.

2.3. Plant extracts as fish anti-pathogenic

Numerous studies have reported a wide range of bioactivities displayed by natural products from plants, fungus and algae, which revealed to be of great interest in the prevention or treatment of pathogens (e.g. Tagboto and Townson, 2001; Zheng et al., 2007; Zahir et al., 2009). Several current studies showed that plant extracts presented anti parasitic

properties both *in vitro* and *in vivo* against fish pathogens (bacteria, virus, fungus, helminths and other parasites) (Table 2).

2.3.1. Antibacterial

2.3.1.1. Tests in vitro

Antibacterial properties of plant products have been by far the most studied bioactivity with potential application in aquaculture systems (Table 2; Table 3). Castro et al. (2008) found that 31 methanolic extracts of Brazilian plants presented antibacterial activity (agar diffusion assay) against the fish pathogenic bacteria *Streptococcus agalactiae*, *Flavobacterium columnare* and *Aeromonas hydrophila*, being *F. columnare* the most susceptible microorganism to most of the tested extracts. Wei and Musa (2008) studied the susceptibility (minimum inhibitory concentration, MIC) of two Gram positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*), four Gram negative bacteria (*Citrobacter freundii*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*) and 18 isolates of *Edwardsiella tarda* to aqueous extract of garlic (500, 250, 125, 62.5 mg/mL), and found that all garlic extracts were effective against the tested pathogenic bacteria.

Some recent studies reveal seaweed and algae as a potential source of antimicrobial products (e.g. Alghazeer et al., 2013; Mendes et al., 2013 ; Al-Saif et al., 2014). Dubber and Harder (2008) showed that methanol extracts of red hornweed (*Ceramium rubrum*) (10 mg dry weight/mL) and hexane extracts of oarweed (*Laminaria digitata*) (31 mg dry weight/mL) evoked strong antibacterial activities against 16 different bacteria tested (marine bacteria and fish pathogenic bacteria). They also showed that Gram-positive marine Bacillaceae were generally more susceptible than Gram-negative marine Vibrionaceae. Another study showed the broad antibacterial activity (agar diffusion assay) of ethanol extract of algae limu kohu (*Asparagopsis taxiformis*) (100 mg/mL) against 9 fish pathogenic bacteria, which was specially strong against *Vibrio alginolyticus* (17.0 ± 1.4 mm), *Vibrio vulnificus* (16.8 ± 1.0 mm) and *Aeromonas salmonicida subsp. salmonicida* (15.0 ± 0.9 mm) (Genovese et al., 2012).

Studied plants	Most effective plants	Type of extract	Parasite species	MIC (mg.mL ⁻¹)	References
	<i>Cariniana legalis</i>		<i>A. hydrophila</i>	0,187	
31 Brazilian plants	<i>Actinostemon concolor, Cariniana legalis, Croton floribundus, Cryptocarya aschersoniana, Merremia tomentosa, Myrcia tomentosa, Siparuna guianensis, Virola sebifera</i>	Methanol	<i>F. columnare</i>	0,094	Castro et al., 2008
10 Iranian medicinal plants	<i>Calyptanthes clusifolia, Merremia tomentosa</i>		<i>S. agalactiae</i>	1,5	
	<i>Heracleum lasiopetalum, Satureja bachtiarica</i>	Ethanol	<i>S. iniae</i>	0,078; 0,039	Pirbalouti et al., 2011
<i>Allium sativum</i>		Water	<i>E. tarda, V. parahaemolyticus, V. vulnificus</i>	7,81	Wei and Musa, 2008
<i>Asparagopsis taxiformis*</i>		Ethanol	<i>Citrobacter freundii, Escherichia coli, S. aureus, S. agalactiae, A. salmonicida, P. damsela, P. piscicida, V. alginolyticus, V. cholerae, V. harveyi, V. parahaemolyticus, V. vulnificus</i>	62,5	Genovese et al., 2012.
<i>Colocasia esculenta</i>		Water	<i>V. alginolyticus, V. cholera, V. harveyi, V. parahaemolyticus, V. vulnificus</i>		Wei et al., 2010
<i>Terminalia catappa</i>		Water	<i>A. hydrophila</i>	0,5	Chitmanat et al., 2005
<i>Laminaria digitata*, Laminaria saccharina*, Himanthalia elongata*, Palmaria palmata*, Enteromorpha spirulina*, Gracilaria corticata*, Enteromorpha compressa*, Ulva fasciata*, Aegiceras corniculatum, Xylocarpus granatum, Aegialitis rotundifolia, Aglaia cucullata, Cynometra iripa</i>	<i>H. elongata*</i>	Methanol	<i>Listeria monocytogenes, Salmonella abony, Enterococcus faecalis, Pseudomonas aeruginosa</i>		Cox et al., 2010
	<i>C. iripa</i>	Methanol	<i>E. tarda, V. alginolyticus, P. fluorescens, P. aeruginosa, A. hydrophila</i>	0,038	Choudhury, et al., 2005
<i>Mastocarpus stellatus*, L. digitata*, Ceramium rubrum</i>	<i>L. digitata*, C. rubrum*</i>	Hexane, methanol	<i>A. hydrophila, A. salmonicida, L. anguillarum, P. damsela, P. anguilliseptica, V. alginolyticus, Yersinia ruckeri</i>		Dubber and Harder, 2008
<i>Gracilaria edulis*, Calorpha peltada*, Hydroclothres sp.*</i>	<i>G. edulis*</i>	Ethanol	<i>E. coli, Enterobacter aerogenes, S. aureus, P. aeruginosa, S. faecalis, B. cereus</i>		Kolanjinathan et al., 2009
<i>Rosmarinus officinalis, Zataria multiflora, Anethum graveolens, Eucalyptus globulus</i>	<i>Rosmarinus officinalis</i>	Essential oil	<i>S. iniae</i>	3,9	Roomiani et al., 2013
<i>Sargassum latifolium*</i>		Chloroform	<i>V. parahaemolyticus, V. harveyi, V. alginolyticus</i>	10 5	Dashtiannasab et al., 2012

Table 2. In vitro anti-bacterial studies of several plant extracts and herbal mixture extracts. * plants are from marine origin (seaweeds or macroalgae).

2.3.1.2. Tests in vivo

Indian major carp (*Labeo rohita*) fed with enriched diets in indian ginseng (*Achyranthes aspera*) (0.2%) and prickly chaff flower (*Whitania somnifera*) (0.5%) showed 41% and 49% respectively of reduced mortality when it was challenged against *A. hydrophila* compared to control groups (Vasudeva-Rao et al., 2006; Sharma et al., 2010). In other studies, tilapia (*Oreochromis mossambicus*) intraperitoneally injected with water extracts of purple fruited pea eggplant (*Solanum trilobatum*) (400 mg/Kg) and chinese cedar (*Toona sinensis*) (8 mg/kg) presented 27% and 57% respectively of reduced mortality when they were challenged against *A. hydrophila* compared to control groups (Divyagnasweri et al., 2007; Wu et al., 2010). Moreover, Divyagnasweri et al. 2007 found that at single herbal extract injection the highest dose (400 mg/kg) of *Solanum trilobatum* water extract exhibited the best protection, but when administered twice, ethanol lowest dose extract (4 mg/kg) presented the lowest mortality (16.7%). Harikrishnan et al. (2012a) observed that kelp grouper (*Epinephelus bruneus*) fed with different doses of chaga mushroom (*Inonotus obliquus*) ethanolic extract had a lower cumulative mortality (20% and 15% for 1% and 2% enriched diet respectively) after 30 days of *Vibrio harveyi* infection compared to control group (90% mortality) (See Table 3).

Plant	Fish	Type of extract	Type of administration	Length of treatment (days)	Growth promoter	Immunostimulant	Antibacterial	References
<i>Achyranthes aspera</i>	<i>Labeo rohita</i>	Powder (seeds)	Oral	35		+	<i>A. hydrophila</i>	Vasudeva Rao et al., 2006
<i>Aegle marmelos</i>	<i>Cyprinus carpio</i>	Powder (leaves)	Oral	50		+	<i>A. hydrophila</i>	Pratheepa et al., 2010
	<i>Labeo rohita</i>	Powder (bulb)	Oral	60		+	<i>A. hydrophila</i>	Sahu et al., 2007
	<i>Lates calcarifer</i>	Powder (bulb)	Oral	14	+	+	<i>V. harveyi</i>	Talpur et al., 2012
<i>Allium sativum</i>	<i>Oreochromis niloticus</i>	Powder (bulb)	Oral	30, 60, 90	+	+	<i>A. hydrophila</i>	Shalaby 2006, Aly and Atti 2008, Aly and Mohamed, 2010
	<i>Onchorrhincus mykiss</i>	Powder (bulb)	Oral	14,58	+	+	<i>A. hydrophila</i>	Nya and Austin, 2009b; Fazlollahzadeh et al., 2011
<i>Andrographis paniculata</i>	<i>Oreochromis niloticus</i>	Powder, water	Oral	14			<i>S. agalactiae</i>	Rattanachaikunsopon and Phumkhaichorn, 2009
<i>Artemisia capillares</i>	<i>Pagrus major</i>	Powder (leaves)	Oral	84	+	+	<i>V. anguillarum</i> <i>V. harveyi</i> , <i>V. alginolyticus</i> , <i>V. parahemolyticus</i> <i>Photobacterium damsela</i>	Ji et al., 2007b
<i>Asparagopsis taxiformis</i>	<i>Penaeus monodon</i>	Organic solvents	Oral	21,28				Manilal et al., 2012
<i>Astragalus membranaceus</i>	<i>Sciaenops ocellatus</i>	Powder	Oral	28		+	<i>V. splendidus</i>	Pan et al., 2013
<i>Azadirachta indica</i>	<i>Lates calcarifer</i>	Powder (leaves)	Oral	15	+	+	<i>V. harveyi</i>	Talpur et al., 2013a
<i>Cinnamomum verum</i>	<i>Oreochromis niloticus</i>	Essential oil	Oral	14			<i>S. iniae</i>	Rattanachaikunsopon and Phumkhaichorn, 2010
<i>Cnidium officinale</i>	<i>Pagrus major</i>	Powder (root)	Oral	84	+	+	<i>V. anguillarum</i>	Ji et al., 2007b
<i>Coriandrum sativum</i>	<i>Catla catla</i>	Powder (leaves)	Oral	14		+	<i>A. hydrophila</i>	Innocent et al., 2011
<i>Crataegi fructus</i>	<i>Pagrus major</i>	Powder (fruit)	Oral	84	+	+	<i>V. anguillarum</i>	Ji et al., 2007b
	<i>Pagrus major (larvae)</i>	Methanol	Oral	12h	+		<i>Vibrio sp.</i>	Takaoka et al., 2011
<i>Curcuma longa</i>	<i>Labeo rohita</i>	Powder (root)	Oral	60		+	<i>A. hydrophila</i>	Sahu et al., 2008
<i>Cynodon dactylon</i>	<i>Catla catla</i>	Ethanol	Oral	60		+	<i>A. hydrophila</i>	Kaleeswaran et al., 2011
<i>Echinacea purpurea</i>	<i>Oreochromis niloticus</i>	Powder	Oral	30, 60, 90	+	+	<i>A. hydrophila</i>	Aly and Mohamed, 2010
<i>Eichhornia crassipes</i>	<i>Macrobrachium rosenbergii</i>	Water	Oral	12		+	<i>Lactococcus garvieae</i>	Chang et al., 2013
<i>Eriobotrya japonica</i>	<i>Epinephelus bruneus</i>	Ethanol	Oral	30		+	<i>V. carchariae</i>	Kim et al., 2011
<i>Euphorbia hirta</i>	<i>Cyprinus carpio</i>	Water	Oral	50		+	<i>P. fluorescens</i>	Pratheepa and Sukumaran, 2011
<i>Excoecaria agallocha</i>	<i>Amphiprion sebae</i>	Methanol	Injection				<i>A. hydrophila</i>	Dayanithi et al., 2012
<i>Fructus forsythiae</i>	<i>Sciaenops ocellatus</i>	Powder	Oral	28		+	<i>V. splendidus</i>	Pan et al., 2013
<i>Gracilaria tenuistipitata</i>	<i>Litopenaeus vannamei</i>	Powder	Oral	35		+	<i>V. alginolyticus</i>	Sirirustananun et al., 2011
<i>Inonotus obliquus</i>	<i>Epinephelus bruneus</i>	Ethanol	Oral	28		+	<i>V. harveyi</i>	Harikrishnan et al., 2012a
<i>Kalopanax pictus</i>	<i>Epinephelus bruneus</i>	Ethanol	Oral	28		+	<i>V. alginolyticus</i>	Harikrishnan et al., 2011e
<i>Lactuca indica</i>	<i>Epinephelus bruneus</i>	Ethanol	Oral	28		+	<i>S. iniae</i>	Harikrishnan et al., 2011c
<i>Massa medicata</i>	<i>Pagrus major</i>	Powder (fruit)	Oral	84	+	+	<i>V. anguillarum</i>	Ji et al., 2007
	<i>Cyprinus carpio</i>	Methanol	Intraperitoneal		+	+	<i>A. hydrophila</i>	Pavaraj et al., 2011
<i>Ocimum sanctum</i>	<i>Oreochromis mossambicus</i>	Water	Intraperitoneal, oral	1, 2, 4		+	<i>A. hydrophila</i>	Logambal et al., 2000
<i>Origanum heracleoticum</i>	<i>Ictalurus punctuatus</i>	Essential oil	Oral	40	+		<i>A. hydrophila</i>	Zheng et al., 2009
<i>Rosmarinus officinalis</i>	<i>Oreochromis sp</i>	Powder, ethyl acetate	Oral	15			<i>S. iniae</i>	Abutbul et al., 2004
<i>Sophora flavescens</i>	<i>Oreochromis niloticus</i>	Ethanol	Oral	30		+	<i>S. agalactiae</i>	Wu et al., 2013
<i>Scutellaria baicalensis</i>	<i>Oplegnathus fasciatus</i>	Ethanol	Oral	42			<i>E. tarda</i>	Harikrishnan et al., 2011f
<i>Siegebeckia glabrescens</i>	<i>Sciaenops ocellatus</i>	Powder	Oral	28		+	<i>V. splendidus</i>	Pan et al., 2013
	<i>Epinephelus bruneus</i>	Ethanol	Oral	28			<i>V. parahemolyticus</i>	Harikrishnan et al., 2012b
<i>Solanum trilobatum</i>	<i>Oreochromis mossambicus</i>	Water, Hexane	Intraperitoneal			+	<i>A. hydrophila</i>	Divyagnaneswari et al., 2007
<i>Terminalia catappa</i>	<i>Beta splendens</i> Regan	Water	Bath				<i>A. hydrophila</i>	Purivirojkul, W. 2012
		Water	Intraperitoneal			+	<i>A. hydrophila</i>	Alexander et al., 2010
<i>Tinospora cordifolia</i>	<i>Oreochromis mossambicus</i>	Ethanol, petroleum ether	Intraperitoneal			+	<i>A. hydrophila</i>	Sudhakaran et al., 2006
<i>Toona sinensis</i>	<i>Oreochromis mossambicus</i>	Water	Intraperitoneal			+	<i>A. hydrophila</i>	Wu et al., 2010
<i>Viscum album</i>	<i>Anguilla japonica</i>	Water	Oral	14		+	<i>A. hydrophila</i>	Choi et al., 2008
	<i>Oreochromis niloticus</i>	Water	Oral	80		+	<i>A. hydrophila</i>	Park and Choi, 2012
<i>Whitania somnifera</i>	<i>Labeo rohita</i>	Powder (root)	Oral	42		+	<i>A. hydrophila</i>	Sharma et al., 2010
	<i>Lates calcarifer</i>	Powder	Oral	15	+	+	<i>V. harveyi</i>	Talpur et al., 2013b
<i>Zingiber officinale</i>	<i>Onchorrhincus mykiss</i>	Water, powder	Oral	1/4	+	+	<i>A. hydrophila</i>	Düğenci, 2003; Nya and Austin 2009a; Haghighi and Rohani, 2013

Table 3. In vivo anti-bacterial studies of several plant extracts and herbal mixture extracts. *Plants are from marine origin (seaweeds or macroalgae).

2.3.2. Anthelmintic (monogeneans)

Monogenean parasites are flatworms which inhabit skin, gills and eventually eyes of fish. Monogeneans from the genus *Dactylogyrus*, *Gyrodactylus* and *Neobenedenia* are widespread parasites affecting a large number of cultured fish and inducing significant economic losses worldwide (Deveney et al., 2001; Woo et al., 2002). Up to now, there are no effective methods to prevent monogenean infections in open aquaculture systems and the only available technique is to remove attached parasite stages through different bath treatments. Moreover, monogenean eggs are highly resistant to physical and chemical treatments due to their sclerotised protein shell protecting the developing embryo (Ernst et al., 2005; Whittington, 2012).

In consequence, several studies have been performed recently to assess the anthelmintic activity of plant extracts in order to treat monogenean infections (Table 4). Plant extracts like methanolic extract of bupleurum root (*Radix bupleuri chinensis*), aqueous and methanolic extracts of cinnamon (*Cinnamomum cassia*), methanolic extract of Chinese spice bush (*Lindera aggregata*) and methanolic and ethyl acetate extracts of golden larch (*Pseudolarix kaempferi*) have shown to possess 100% *in vivo* efficacy against monogenean *Dactylogyrus intermedius* when added to water of infected goldfish (*Carassius auratus*) (Wu et al., 2011; Ji et al., 2012). Tu et al. (2013) studied the effect of several extracts of indian sandalwood (*Santalum album*) against *Dactylogyrus intermedius* and *Gyrodactylus elegans* on goldfish and found that chloroform extract was the most effective and safest for the fishes. They also observed that bath treatment with long duration and multiple administrations could eliminate a greater proportion of monogenean infections.

Hutson et al. (2012) assessed the effect of aqueous extracts from different algae on the lifecycle of the parasite *Neobenedenia* sp. They observed that infection success on *Lates calcarifer* was lower in the presence of *Asparagopsis taxiformis* (51%) and *Ulva* sp. (54%) extracts in seawater compared with the control (71%). Moreover, *A. taxiformis* extract inhibited embryonic development of *Neobenedenia* sp. and reduced hatching success to 3% compared with 99% for the seawater control. Militz et al. (2013 a,b) fed farmed barramundi (*L. calcarifer*) with two enriched garlic diets and observed that when fish were challenged with *Neobenedenia* sp. treated groups during long-term supplementation (30 days) displayed

70% reduced infection success compared to control and short-term supplementation (10 days).

Plant	Fish	Type of extract	Type of administration	Anthelmintic	References
		Water (bulb)	Oral	<i>Neobenedenia</i> sp.	Militz et al., 2013 a,b
	<i>Lates calcarifer</i>				
<i>Allium sativum</i>		Water (bulb)	Bath	<i>Neobenedenia</i> sp.	Militz et al., 2013 a,b
	<i>Piaractus mesopotamicus</i>	Powder (bulb)	Oral	<i>Anacanthorus penilabiatus</i>	Martins et al., 2002
<i>Artemisia annua</i>	<i>Heterobranchius longifilis</i>	Ethanol (leaves)	Bath	Monogean	Ekanem and Brisibe, 2010
<i>Artemisia argyi</i>	<i>Carassius auratus</i>	ME, PE, CHL, <u>EA</u> , water	Bath	<i>D. intermedius</i>	Huang et al., 2013
<i>Asparagopsis taxiformis</i> *	<i>Lates calcarifer</i>	Water	Bath	<i>Neobenedenia</i> sp.	Hutson et al., 2012
<i>Caesalpinia sappan</i>	<i>Carassius auratus</i>	ME, PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Huang et al., 2013
<i>Chelidonium majus</i>	<i>Carassius auratus</i>	Ethanol	Bath	<i>D. intermedius</i>	Yao et al., 2011
<i>Cimifuga foetida</i>	<i>Carassius auratus</i>	ME, PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Wu et al., 2011
<i>Cinnamomum cassia</i>	<i>Carassius auratus</i>	<u>ME</u> , PE, CHL, EA, <u>water</u>	Bath	<i>D. intermedius</i>	Ji et al., 2012
<i>Cuscuta chinensis</i>	<i>Carassius auratus</i>	ME, PE, CHL, <u>EA</u> , water	Bath	<i>D. intermedius</i>	Huang et al., 2013
<i>Dryopteris crassizhizoma</i>	<i>Carassius auratus</i>	<u>ME</u> , PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Lu et al., 2012
<i>Eupeptorium fortunei</i>	<i>Carassius auratus</i>	ME, PE, <u>CHL</u> , EA, water	Bath	<i>D. intermedius</i>	Huang et al., 2013
<i>Kochia scoparia</i>	<i>Carassius auratus</i>	<u>ME</u> , PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Lu et al., 2012
<i>Lindera aggregata</i>	<i>Carassius auratus</i>	<u>ME</u> , PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Ji et al., 2012
<i>Lysima chiachristinae</i>	<i>Carassius auratus</i>	ME, PE, CHL, <u>EA</u> , water	Bath	<i>D. intermedius</i>	Huang et al., 2013
<i>Momordica cochinchinensis</i>	<i>Carassius auratus</i>	ME, PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Wu et al., 2011
<i>Piper guineense</i>	<i>Carassius auratus</i>	Methanol (seeds)	Oral	Monogean	Ekanem et al., 2004b
<i>Polygala tenuifolia</i>	<i>Carassius auratus</i>	PE, CHL, EA, <u>acetone</u> , <u>ME</u>	Bath	<i>D. intermedius</i>	Lu et al., 2012
<i>Prunus amygdalus</i>	<i>Carassius auratus</i>	ME, PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Wu et al., 2011
<i>Pseudolarix kaempferi</i>	<i>Carassius auratus</i>	<u>ME</u> , PE, CHL, EA, water	Bath	<i>D. intermedius</i>	Ji et al., 2012
<i>Radix Bupleuri chinense</i>	<i>Carassius auratus</i>	PE , CHL, EA, ME, water	Bath	<i>D. intermedius</i>	Wu et al., 2011
<i>Radix peucedani</i>	<i>Carassius auratus</i>	PE , CHL, EA , ME , water	Bath	<i>D. intermedius</i>	Wu et al., 2012
<i>Rhizoma cimicifugae</i>	<i>Carassius auratus</i>	PE , CHL, EA, ME, water	Bath	<i>D. intermedius</i>	Wu et al., 2012
				<i>D. intermedius</i> ,	
<i>Santalum album</i>	<i>Carassius auratus</i>	<u>CHL</u> , ME, EA, water	Bath	<i>Gyrodactylus elegans</i>	Tu et al., 2013
<i>Semen momordicae</i>	<i>Carassius auratus</i>	PE, CHL , EA , ME , water	Bath	<i>D. intermedius</i>	Wu et al., 2012
<i>Semen amygdali</i>	<i>Carassius auratus</i>	PE , CHL, EA, <u>ME</u> , water	Bath	<i>D. intermedius</i>	Wu et al., 2013
<i>Ulva sp.</i> *	<i>Lates calcarifer</i>	Water	Bath	<i>Neobenedenia</i> sp.	Hutson et al., 2012

Table 4. In vivo anti-helminth studies of several plant extracts and herbal mixture extracts. * plants are from marine origin (seaweeds or macroalgae). **PE**: Petroleum ether, **CHL**: chloroform, **EA**: Ethyl acetate, **ME**: Methanol. Underlined solvents are the solvents that displayed better results, while bold letter solvents didn't show any activity.

2.3.3. Other parasites

Until now only few studies have reported plant antiviral, antifungal or antiprotozoal activities on fish pathogens (Table 5).

Dierebusarakom et al. (1996) found that 18 Thai traditional herbs presented antiviral activity against Oncorhynchus Mason Virus (OMV) and Infectious Haematopoietic Necrosis Virus (IHNV), while *Orchocarpus siamensis* and star gooseberry (*Phyllanthus acidus*) inhibited the

replication of OMV and IPNV (Infectious Pancreatic Necrosis Virus) in cells. Dierebusarakom et al. (1998) showed that *Clinacanthus nutans* ethanolic extract inactivated Yellow-Head Virus (YHV) *in vitro* at concentrations as low as 1 µg/mL, and when black tiger shrimp (*Penaeus monodon*) were fed with the extract, the cumulative mortality decreased from 75% in control group to 33% in 1% enriched diet. In other studies *P. monodon* challenged with White Spot Syndrome Virus (WSSV) and intramuscular injected or orally administered aqueous extract of Bermuda grass displayed no mortality and no signs of white spot disease compared to 100% mortality observed in control group (Balasubramanian 2007, 2008a and 2008b). All these studies point out that virus inactivation may occur by the interaction between the extract and the envelope of the virus.

Xue-Gang et al. (2013) showed that 10 plant species used in traditional Chinese medicine displayed strong inhibition against *Saprolegnia* and *Achlya klebsiana*, being the petroleum ether extracts of conidium fruit (*Cnidium monnieri*), magnolia bark (*Magnolia officinalis*) and aucklandia root (*Aucklandia lappa*) the ones with the best antifungal activity. Hashemi-Karouei et al. (2011) found that ethanol extracts of common rue (*Ruta graveolens*) had anti fungal effects and prevented growth of *Saprolegnia sp.*, while Genovese et al. (2013) found that the red algae *Asparagopsis taxiformis* presented antifungal activity against *Aspergillus* species. In an *in vivo* study, Chitmanat et al. (2005) showed that indian almond leaves (*Terminalia catappa*) extract reduced the fungal infection in tilapia eggs.

Buchmann et al. (2003) found that garlic extract killed theronts of the ciliate *Ichthyophthirius multifiliis* responsible for the white spot disease at 62.5 mg/L and tomocysts of the same parasite at 570 mg/L. Harikrishnan et al. (2012c) showed that mortality of infected olive flounder by the ciliate *Miamiensis avidus* decreased from 80% in the control diet to 40% when fish were fed with a supplemented diet in *Suaeda maxima* extract.

These studies highlight the antiparasitic activities of numerous plant extracts and their potential to be used as an alternative to chemical treatments. However, the use of natural products in aquaculture is a very recent trend so even if most of the studies show promising results there is still little studies who have analyzed the long-term effects on fish physiology. Several studies have shown that plant extracts can have a toxic effect on fish if applied at inadequate doses (e.g. Sambasivam et al., 2003; Ekanem et al., 2007 ; Kavitha et al. 2012). Consequently, further regulation and standardization is required in order to use plant products in the aquaculture industry. Normalized extraction procedure depending on the bioactive

molecules identified, application procedure, doses and duration of treatment depending on the type of parasite should be established.

Studied plants	Most effective plants	Type of extract	Parasite species	MIC (mg/mL)	References
20 indian traditional medicinal plants	<i>Aegle marmelos, Cynodon dactylon, Lantana camara, Mormodica charantia, Phyllanthus amarus</i>	Petroleum ether, benzene, diethyl ether, chloroform, ethyl acetate, methanol, ethanol, water	White Spot Syndrome Virus		Balasubramanian et al., 2007
30 medicinal plants	<i>M. officinalis, Sophora alopercuroides</i>	Methanol	<i>I. multifiliis</i>		Yi et al., 2012
24 crude plant extracts	<i>Rumex obtusifolius, Sophora flavescens, Echinacea purpurea and Zingiber officinale</i>	Ethanol	<i>Saprolegnia australis</i>		Caruana et al., 2012
40 plant extracts	<i>Angelica pubescens, Magnolia officinalis, Aucklandia lappa</i>		<i>Saprolegnia</i> sp., <i>Achlya klebsiana</i>	62,5	Xue-Gang et al., 2013
<i>A. taxiformis*</i> <i>Morus alba</i>		Ethanol Acetone, ethyl acetate	<i>Aspergillus</i> sp. <i>I. multifiliis</i>	0,15-5	Genovese et al., 2013. Fu et al., 2014
<i>Ruta graveolens</i>		Ethanol	<i>Saprolegnia</i> sp.	25	Hashemi-Karouei et al., 2011

Table 5. In vitro anti-parasitic studies of several plant extracts and herbal mixture extracts. * plants are from marine origin (seaweed or macroalgae).

3. Isolated natural products with anti parasitic properties

As the interest in the use of plant products in aquaculture is relatively recent, most of the efforts have been made in identifying potential activities on different plant species. However, identifying the active molecules responsible for the observed bioactivities is the next step and would allow 1) to optimize the extraction procedure 2) enable appropriate dosage and 3) study mechanisms of action.

A few isolated and characterized natural products have been evaluated for their activity in preventing and treating disease outbreaks in aquaculture. Kumar et al. (2013) administered oral azadirachtin (**1**) to goldfish and observed that all immunologic parameters were enhanced ($p < 0.05$) as well as hematologic parameters and fish presented a higher relative rate survival (42.6%) compared with the control against *A. hydrophila* infection. The same molecule also displayed potential to treat *Argulus* sp. infection in goldfish, presenting a therapeutic index (difference in toxicity between the parasite and the fish) of 4.10 (Kumar et al., 2012b). Azadirachtin is a highly oxygenated triterpenoid isolated from neem (*Azadirachta indica*) known to have antimicrobial and pesticidal properties while being non-toxic to vertebrates (Govindachari et al., 2000). Some works indicated that azadirachtin block microtubule

formation and thus inhibiting cell division, and other studies indicated that azadirachtin antiparasitic effect can be explained by its effect on molting and juvenile hormones (Mitchell et al., 1997; Mordue and Blackwell, 1993) (Figure 2).

Kumar et al. (2012a) evaluated the effect of piperine (**2**) on the ectoparasite *Argulus sp.* parasitizing *C. auratus* and found that *Argulus* mortalities were positively correlated with concentration of piperine, whose therapeutic index was 5.8. This study confirmed that increase in time of exposure of parasitized fish to compound led to drastic reduction in the parasitic load to a minimum level, confirming that low dosage of piperine can effectively control ectoparasites from fish as long as the duration of exposure to the treatment is increased. Piperine is a bioactive amide isolated from long pepper (*Piper longum*) known to have insect repellent activity (Traxler, 1971) (Figure 2).

Several natural products such as sanguinarine (**3**), chelerythrine (**4**), chelidonine (**5**), dioscin (**6**), polyphyllin D (**7**), brucein A and D (**8,9**), palmitic acid (**10**), pharnilatin (**11**), osthol (**12**) and isopimpinellin (**13**) have been isolated from different plants through bioassay-guided fractionations and proved to have better anthelmintic activity against *D. intermedius* than positive controls of mebendazole (a commercial anthelmintic drug) (Wang et al., 2008, 2010a, 2010b, 2011; Li et al., 2011; Yao et al., 2011; Hao et al., 2012, Liu et al., 2012) (Figure 2). Sanguinarine (**3**) is a benzo[c]phenanthridine alkaloid isolated from Kelway's plume poppy (*Macleaya microcarpa*) that has the ability to cause DNA single and double strand break resulting in DNA damage, which may be responsible for the anthelmintic activity (Matkar et al., 2008). Chelerythrine (**4**) and chelidonine (**5**) are also benzo[c]phenanthridines with antimicrobial, antifungal and anti-inflammatory activity isolated from greater celandine (*Chelidonium majus* L.) (Walterova et al., 1995; Malikova et al., 2006). Chelerythrine (**4**) exhibits high cytotoxic potency against a wide number of cells, through multiple apoptosis-inducing pathways, so a direct action on mitochondria may be involved in the eradication of parasites (Slaninova et al., 2001; Kémeny-Beke et al., 2006; Kaminsky et al., 2008). Some studies like Philchenkov et al. (2008) indicated that the mechanism of action of chelidonine (**5**) against *D. intermedius* may be through the mitochondrial cell death pathway activated through the caspase-9.

Dioscin (**6**) and polyphyllin D (**7**) are two diosgenyl saponins isolated from Himalayan paris (*Paris polyphylla*) that display antibacterial (Hufford et al., 1988), antiviral (Ikeda et al.,

2000), antifungal (Takechi et al., 1991; Sautour et al., 2004), inflammatory (Kim et al., 1999) and high cytotoxic activities (Mimaki et al., 2001; Cheung et al., 2005). Recent works found that mode of action of saponins is similar to that of conventional anthelmintic drugs such as praziquantel, so they would affect the permeability of the cell membrane of the parasites and cause vacuolization and disintegration of monogenea teguments (Schmahl and Taraschewski, 1987; Wang et al., 2010a). Brucein A and D (**8,9**) are two quassinoids isolated from (*Brucea javanica*) that have displayed activity against several protozoan parasites (O'Neill et al., 1987; Wright et al., 1988, 1993; Nakao et al., 2009) as well as anti-inflammatory, antitrypanosomal and antimalarial activities (Hall et al., 1983; Saxena et al., 2003; Bawm et al., 2008) and it seems that their major cellular target on parasites are proteins (Guo et al., 2005).

Synergistic effect of mixed herbal extracts has been reported before, so it is of great importance to determine whether the bioactivity observed is due to isolated molecules or it is rather a consequence of a synergistic effect between several molecules contained in the extracts (Bhuvanewari and Balasundaram, 2006; Ji et al., 2007b; Harikrishnan et al., 2009a, 2010). For example, Lee et al. (2009) showed that the minimum inhibitory concentration (MIC) of clove (*Syzygium aromaticum*) essential oil against bacteria isolated from aquaculture sites was lower than MIC from the supposed bioactive molecule eugenol (**14**) (Figure 2). However, Rattanachaiakunsopon and Phumkhachorn (2010) and Abd el-Galil and Hashiem (2012a) showed that antibacterial activity of carvacrol (**15**) against *Edwardsiella tarda* and *Tenacibaculum maritimum* was enhanced when administered with cymene (**16**) (Figure 2).

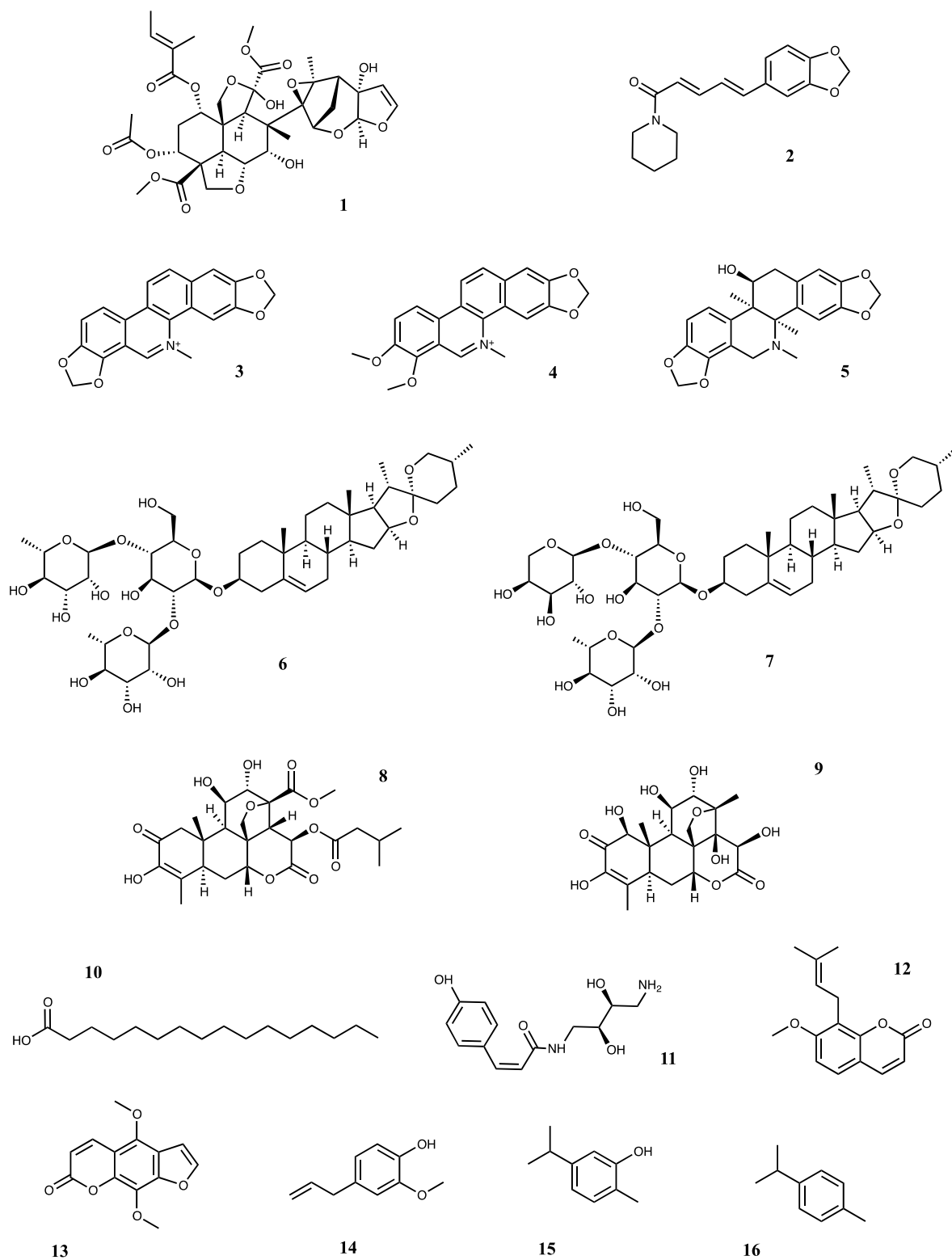


Figure 2. Structures of natural products with anti-parasitic activity with their stereochemistry.

4. Conclusion and perspectives

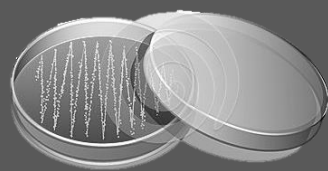
The beneficial properties and efficacy of plant products on the health of cultured fish depends on the plant part, the method of extraction and the extract concentration. Although numerous studies have reported multiple activities and potential application of plant extracts in aquaculture, there has been little effort to homogenize the extraction procedure, the extract concentration and the administration way. Intraperitoneal injection has proved to be the most rapid and efficient way of administration although it is expensive, laborious and stressful for fish, specially for very young specimens; thus oral administration seems the most suitable for aquaculture (Blazer, 1992; Anderson, 1992; Yoshida et al., 1995). Besides, the effect of plant products on fish is dose-dependent and there is a potential for overdosing, so determining the suitable extract concentration is of great importance (Kajita et al., 1990, Harikrishan et al., 2011a). Therefore, the upcoming necessities are quantifying and characterizing chemically plant extracts in order to identify active molecules responsible for the observed activity and thus facilitate the establishment of a standardized protocol including different methods of extraction depending on the bioactive molecules observed, adequate extract concentration and regularized administration way.

Terrestrial plants have been studied and exploited extensively over centuries for the bioactivities of their extracts. Algae are also considered to be a rich source of bioactive molecules, but only recent researches have started to study their chemical composition and to evaluate their potential bioactivities, mainly against human pathogens (e.g. Lima-Filho et al., 2002 ; Sukoso et al., 2012 ; Zbakh et al., 2012). Nevertheless, they present an enormous potential to be used in aquaculture as immunostimulant and preventing diseases outbreaks as 1) they present a wide range of original bioactive molecules (e.g. Cannell, 2006; Holdt and Kraan, 2011; Stengel et al., 2011) they seem to possess multiple strong bioactivities such as antibacterial and anthelmintic (e.g. Mayer and Lehmann, 2000; Al-Saif et al., 2014) and 3) the utilization of local algae can avoid the introduction of exogenous molecules in the marine environment. Therefore, more studies should be conducted in this discipline, following the same directions that plants in order to assure a regulated and appropriate use of either plant or algae extracts in aquaculture.

Regarding treatment of ectoparasites like monogeneans, studies have frequently focused on finding extracts or chemical compounds with anthelmintic properties which can be added

on water to treat infectious stages. However, natural products are not necessarily present in the marine environment and thus their release in the environment (through bath treatment) can have the same side effects than current synthetic drugs (resistance development or side-effects on surrounding wildlife). Hence, a better approach would be to focus on the immunostimulant and anthelmintic properties of these compounds when administered orally (with food) to fish in order to avoid monogenean infection through a reenforced protection of fish against pathogens like the study conducted by Militz et al. (2013 a,b).

Based on this review, it is clear that the use of plant or algae extract can open promising perspectives in terms of parasite treatment in aquaculture. However, it is necessary to carry out tests under homogeneous conditions with controlled amount of identified active molecules along with negative controls for a real estimation of the effectiveness of the tested product. Finally, it seems important not only to determinate the molecules involved in the activity against pathogens but also to establish the induced response on the fish physiology. Quantifying the response in fish can be achieved by measuring hematological indices, but also by analyzing the expression of genes directly or indirectly involved in the physiological response like innate immunity genes.



CHAPTER VIII

Antibacterial activity of local Polynesian plants and algae against fish pathogenic bacteria

Antibacterial activity of local Polynesian plants and algae against fish pathogenic bacteria

Abstract

Bacterial disease outbreaks cause elevated mortality episodes in aquaculture, causing high annual economic losses. The intensive use of antibiotics to prevent and control bacterial outbreaks presents numerous disadvantages including the emergence of resistance drug strains or antibiotic accumulation in animals muscle. Therefore, there is an urgent need of novel sustainable strategies for the control of bacterial infections in aquaculture. In this study the antibacterial activity of several local Polynesian plants (garlic –*Allium sativum*, turmeric – *Curcuma longa*, ginger –*Zingiber officinalis*, noni - *Morinda citrifolia* and tamanu - *Calophyllum inophyllum*) and algae (*Anabaena torulosa*, *Asparagopsis taxiformis*, *Dichotomaria marginata*, *Lyngbya majuscula*, *Padina boryana* and *Turbinaria ornata*) were evaluated against the fish pathogenic bacteria *Tenacibaculum maritimum* and *Vibrio harveyi*. *A. taxiformis* revealed the strongest antibacterial activity against both bacteria. Therefore *A. taxiformis* extract was subjected to fractionation and antibacterial assays coupled to HPLC extract separation in order to localize the antibacterial activity in the extract chromatogram. Purification of the fraction exhibiting a potent antibacterial activity and characterisation of the bioactive compound are in progress in the laboratory. Garlic and turmeric also displayed some activity against *V. harveyi*, however this activity was not found against *T. maritimum*.

Keywords: *Asparagopsis taxiformis*, *Tenacibaculum maritimum*, antibacterial activity, algae extract

1. Introduction

Bacterial diseases present a major constraint to aquaculture development (Deifordt et al. 2011). Opportunistic and pathogenic bacteria are responsible for elevated mortality rates and decreased efficiency in aquaculture, causing high economic losses to fish farmers (Subasinghe et al. 2001; Sitja-Bobadilla et al. 2007). Antibiotics (such as oxytetracycline, chloramphenicol and erythromycin) are widely used to prevent bacterial diseases in fish. However, continuous and intensive use of antibiotics can lead to the emergence of drug resistant strains, leading to reduced efficacy of the drugs. Besides, antibiotic resistance can be transferred by horizontal gene transmission to environmental and human pathogenic bacteria, creating serious public health problems (Cabello et al. 2013).

Therefore, novel strategies to control bacterial infections are needed for a further sustainable development of the aquaculture industry. Prevention of bacterial outbreaks is one of the most important health management options. However, since not all infections can be prevented, new techniques controlling pathogenic bacteria are also needed (Defoirdt et al. 2011). Several biocontrol strategies have been proposed such as the use of probiotic bacteria, bacteriophages that kill specifically pathogenic bacteria or inhibition of bacteria virulence by quorum sensing disruption (Verschuere et al., 2001; Nakai and Park, 2002; Natrah et al. 2011). However, most of these biocontrol strategies are still in the research phase and are not yet applicable to commercial aquaculture facilities (Qi et al. 2009; Defoirdt et al. 2011). The use of medicinal plants in aquaculture has also been proposed as an alternative to chemotherapy (Reverter et al. 2014).

Rural fish farmers around the world have long been using medicinal plants to improve water quality, reduce fish stress and prevent and treat fish diseases (Caruso et al. 2013). Plants and algae possess a wide variety of metabolites that confer them numerous biological activities such as antibacterial, antiviral, antiparasitic and antifungal properties. Numerous plants have been reported to favour various activities like anti-stress, growth promotion, appetite stimulation, and immunostimulation in fish and shellfish (Reverter et al. 2014). The use of plants in aquaculture could therefore enhance fish fitness and immunity, decreasing risk of bacterial outbreaks but also contribute to bacteria control in infections due to their antibacterial properties. Most investigations have focused on the study of terrestrial plants,

however recent works show that algae possess structurally novel and biologically active metabolites with interesting activities on fish pathogens (Sukoso et al. 2012; Zbakh et al. 2012).

Vibrio harveyi (Vibrionaceae) is Gram-negative luminous bacteria recognized as an important cause of disease in fish and marine invertebrates like penaeid shrimps. In fish, diseases include vasculitis, gastro-enteritis and eye lesions, while in shrimp *V. harveyi* is associated with luminous vibriosis (the shrimp glow in the dark, Prayitno and Latchford, 1995), *Bolitas negricans* (diseased animals contain balled epidermal tissue blocking the digestive tract, Robertson et al. 1998) and bacterial white tail disease (Austin and Zhang, 2006; Zhou et al. 2012a). Recent studies have reported that several *V. harveyi* strains display multiple resistance against antibiotics like cephalothin, vancomycin, ampicillin, cefepime, cefotetan, ciprofloxacin, penicillin, rifampicin and streptomycin, indicating the urgent need for targeted alternative biocontrol strategies in aquaculture (Kang et al. 2014; Stalin and Srinivasan, 2016). Several medicinal plants (e.g. garlic-*Allium sativum* and ginger – *Zingiber officinalis*) and algae (e.g. *Asparagopsis taxiformis* and *Sargassum latifolium*) have been reported to display antibacterial activity against some *V. harveyi* strains (Dashtiannasab et al. 2012; Genovese et al. 2012; Talpur and Ikhwanuddin; 2012; Talpur et al. 2013).

Tenacibaculum maritimum (Flavobacteriaceae) is also a Gram-negative bacteria with worldwide distribution responsible for the ulcerative disease tenacibaculosis, which causes important losses in fish aquaculture. Tenacibaculosis causes mainly lesions on the body surface of fish such as ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes (Ostland et al. 1999; Avendaño-Herrera et al. 2006b). Most tenacibaculosis treatments to date include the use of antibiotics, however, studies indicated that repetitive therapy with the same antimicrobial treatment resulted in *T. maritimum* resistance (Avendaño-Herrera et al. 2007). However, to date only one study has assessed the effect of natural products (carvacrol and cymene, both major compounds in oregano and thyme) on *T. maritimum* (Abd-el-Galil et al. 2012a).

In this chapter, the antibacterial activity of several local plant or easily found in French Polynesia (garlic –*Allium sativum*, turmeric – *Curcuma longa*, ginger –*Zingiber officinalis*, noni - *Morinda citrifolia* and tamanu - *Calophyllum inophyllum*) and algae (*Anabaena torulosa*, *Asparagopsis taxiformis*, *Dichotomaria marginata*, *Lyngbya majuscula*, *Padina*

boryana and *Turbinaria ornata*) were evaluated against the fish pathogenic *Tenacibaculum maritimum* and *Vibrio harveyi*. After a first antibacterial evaluation, the most active extract (*A. taxiformis*) was subjected to fractionation and antibacterial assays coupled to HPLC extract separation were performed in order to identify the bioactive molecules responsible for the observed antibacterial activity.

2. Material and methods

2.1. Extracts preparation for the biological activity screening

Fresh plant material (garlic –*A. sativum*, ginger –*Z. officinalis*, and turmeric –*C. longa*) and tamanu essential oil (*C. inophyllum*) were bought from the local market in Moorea in April 2014 (French Polynesia). Noni (*M. citrifolia*) was collected in the Moorea rainforest in April 2014, whereas red algae (*A. taxiformis* and *D. marginata*) and cyanobacteria (*A. torulosa* and *L. majuscula*) were collected on the reef outer slope and the brown algae (*T. ornata* and *P. boryana*) in the fringing reef in Moorea in April 2014. *A. taxiformis* was also collected in February 2015; June 2015 and May 2016. Noni and the algae (*A. taxiformis*, *D. marginata*, *A. torulosa*, *L. majuscula*, *T. ornata* and *P. boryana*) as well as peeled garlic, ginger and turmeric bulbs were freeze-dried, powdered and stored at -20°C until used.

Plant extracts were prepared extracting 3 times approximately 1g of dried mass with 20 mL of CH₂Cl₂-MeOH (1:1) in an ultrasonic bath for 10 min. Extracts were evaporated by vacuum rotatory evaporation (40°C), freeze-dried and stored at -20°C until used.

Algae samples (approximately 1g) were extracted the same way (1:1 CH₂Cl₂-MeOH) but prior to evaporation 500 mg of octadecyl silica powder were added. Extracts were evaporated to dryness by vacuum rotatory evaporation, and loaded onto RP18 Solid Phase Extraction (SPE) cartridges (Phenomenex, CA, USA). The conditioned cartridges were first washed with 10 mL of H₂O in order to remove the salts and then with 5 mL of CH₂Cl₂-MeOH (1:1). Water soluble fractions were discarded and CH₂Cl₂-MeOH fractions were dried under nitrogen and stored at -20°C until used.

Fractionation of the most bioactive extract (*A. taxiformis*) was performed. The algae *A. taxiformis* (2 g) was extracted as described above and the conditioned cartridges were washed with 20 mL of H₂O and then with 20 mL of MeOH (A), MeOH- CH₂Cl₂ (B), and CH₂Cl₂ (C) to obtain three fractions (A, B and C).

2.2. Antibacterial tests

2.2.1. Broth dilution tests

A first evaluation of the antibacterial activities of plants and algae extracts was performed with broth dilution tests and disk diffusion assays. Broth dilution tests of plant and algae extracts were performed against the pathogenic bacteria *Tenacibaculum maritimum* and *Vibrio harveyi*. Bacterial strains *T. maritimum* (TFA4 isolated from *P. orbicularis* from Tahiti, French Polynesia, Centre IFREMER du Pacifique, Tahiti) and *V. harveyi* (LMG4044, Centre IFREMER du Pacifique, Tahiti) were cultured 24 hours prior to *in vitro* tests in Zobell Marine Broth 2216 at 28°C. 20 µL of plant or algae extract diluted in DMSO and 180 µL of 1/25 diluted bacterial solution (*T. maritimum* or *V. harveyi*) were added to 96-well microplates. Plant and algae extracts were tested at 3 final concentrations: 2 mg/mL, 0.2 mg/mL and 0.02 mg/mL. Negative controls were done replacing the bacterial solution by Zobell Marine broth and positive controls were done with 20 µL of DMSO (1, 0.1, 0.01%) and 180 µL of bacterial solution. Triplicates were performed for all samples and controls. Absorbance ($\lambda = 340$ nm) was monitored with a FLUOStar Optima microplate spectrophotometer during 6 hours (36 cycles, 10 min/cycle).

2.3.2. Disk diffusion essays

Disk diffusion assays were used as a first screening of bioactive plants and algae against the fish pathogenic bacteria *T. maritimum* (Aq9-64 isolated from *P. orbicularis* from Tahiti, Labofarm Laboratories, Loudeac, France). 20 µL of plant or algae extract diluted in MeOH (25 mg/mL) were imbibed in Whatman filters (6 mm diameter) and placed under an extractor hood for solvent evaporation. Positive controls consisted of disks imbibed with 20 µL of erythromycin solution (2.5 mg/mL) and negative controls consisted of disks imbibed with 20

μL of MeOH. Bacteria was cultured 24 hours prior to *in vitro* tests in LB Broth (5 g/L yeast extract (Simca), 10 g/L peptone (Simca) and 20 g/L NaCl) at 28°C and diluted at 1/25 (1 mDo). 100 μL of bacterial culture were added on agar plate surfaces and spread homogeneously with sterile glass beads. The impregnated disks (plant or algae extract, positive control and negative control) were placed on the agar plates suitably spaced apart and the plates were incubated at 28°C. Inhibition zones (IZ) were measured after 24 hours of incubation. Triplicates for each extract were performed and the results (inhibition efficiencies) were expressed as the triplicate's mean of the division between the extract inhibition zone and the erythromycin inhibition zone ($IE = IZ_{\text{extract}} / IZ_{\text{erythromycin}}$). Disk diffusion assays were also performed to evaluate the antibacterial capacity of the three *A. taxiformis* fractions. Fractions A, B and C and were diluted in MeOH (50 mg/mL) and disk diffusion assays were performed as described above.

2.3.3. Antibacterial assays coupled to HPLC extract separation.

Complementary antibacterial tests of *A. taxiformis* methanolic extract (fraction B) were done on 96-well plates previously coupled to HPLC fractionation in order to localise the extract bioactivity on a region or regions of the chromatogram. Antibacterial activity was evaluated on *T. maritimum* (Aq9-64). The experiment was carried out on a system from Waters including Alliance separation module 2695, the 2998 photodiode array detector, the 2424 ELS detector and a Fraction Collector III (WFC III) equipped with a rack supporting 96 well microtiter plates (Waters Corp. MA, USA). The equipment was controlled, and the data was handled by the Empower Chromatography Data Software. HPLC elution conditions consisted of two eluants, A (water with 1‰ formic acid) and B (acetonitrile with 1‰ formic acid), an elution profile based on a linear gradient from 15 % to 100 % B in from 5 to 25 min, a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$, and an injection volume of 20 μL of an extract at 50 mg/mL. Routine detection was set at 254 nm, and the column used was Gemini C6-Phenyl (250 x 3 mm, 5 μ , Phenomenex). From the column outlet, the eluate was divided, 0.3 mL/min were analyzed by PDA and ELS detectors, and 0.7 mL/min were collected continuously at uniform volume intervals (262 $\mu\text{L}/\text{well}$) in 96-well microplates.

T. maritimum was cultured 24 hours prior to *in vitro* tests in LB Broth at 28°C. Five µl of DMSO (to dissolve the metabolites) and 195 µL of 1/25 diluted bacterial solution (1 mDO) were added to freeze-dried microplates. Six positive controls were done by microplate (three replicates). Microplates were incubated at 28 °C for 24 h. Bacterial growth was estimated reading optical absorbance at 630 nm with a microplate reader Aviso Sirius HT (Ebersberg, Germany), after inoculation and 24 h later and % of bacteria growth inhibition were calculated.

3. Results

3.1. Antibacterial screening of plant and algae extracts

3.1.1. Broth dilution tests

Broth dilution tests showed that the highest dose (2 mg/mL) of *A. sativum*, *C. longa* and *A. taxiformis* (2014) inhibited growth of *V. harveyi*. Partial growth inhibition of *V. harveyi* was observed at weaker concentrations of *C. longa* extract (0.2, 0.002 mg/mL), revealing a dose-effect relationship. No bacterial growth inhibition was observed with any of the other plants (*M. taxifolia*, *C. inophyllum* and *Z. officinalis*) and algae (*A. torulosa*, *D. marginata*, *L. majuscula*, *P. boryana* and *T. ornata*) at any of the three concentrations studied (Figure 1).

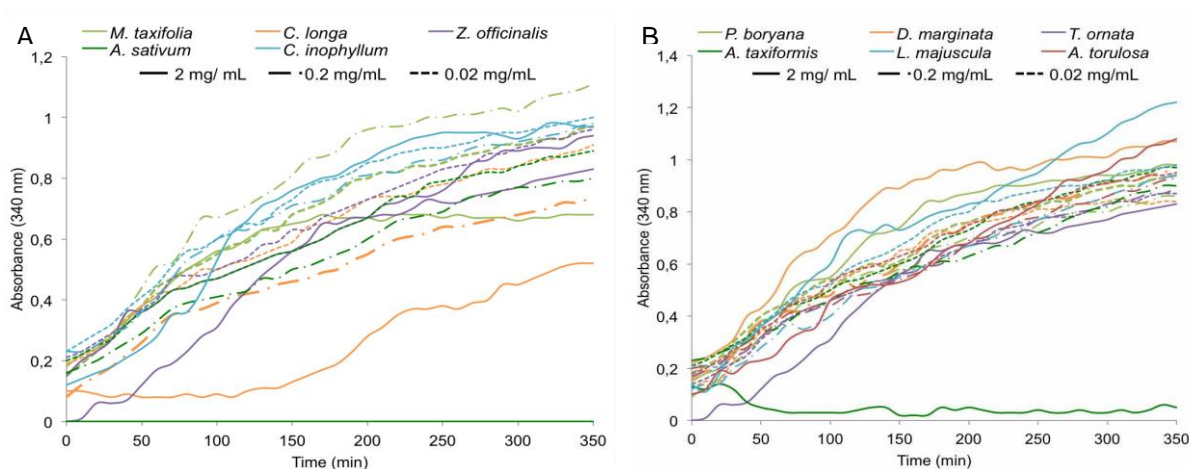


Figure 1. *V. harveyi* growth curves ($\lambda = 340$ nm) with plant (A) and algae (B) extracts at three concentrations (2, 0.2 and 0.02 mg/mL).

T. maritimum agglutinated during broth dilution tests forming filaments, and no bacterial exponential growth was observed after 6 hours of incubation. Disk diffusion assays were done to evaluate plant and algae antibacterial activities against *T. maritimum*.

3.1.2. Disk diffusion assays

Disk diffusion assays revealed that *A. taxiformis* (2014) was the only active extract against *T. maritimum* (IE = 0.45 ± 0.22) at the concentration tested (500 μ g). None of the other plants or algae (including garlic and turmeric) displayed any antibacterial activity on *T. maritimum*.

3.2. Antibacterial activity of *A. taxiformis* fractions

Antibacterial activity of *A. taxiformis* (2014) fractions A (g) and B (g) was tested with disk diffusion assays (20 μ L of 50 mg/mL extract) against the least susceptible bacteria *T. maritimum*. C fraction (CH_2Cl_2) yielded approximately 1 mg of dried extract, so antibacterial activity could not be evaluated for this fraction. Results show that only the A fraction (MeOH) presents strong antibacterial activities against *T. maritimum* (3.01 ± 0.53) (Figure 2).

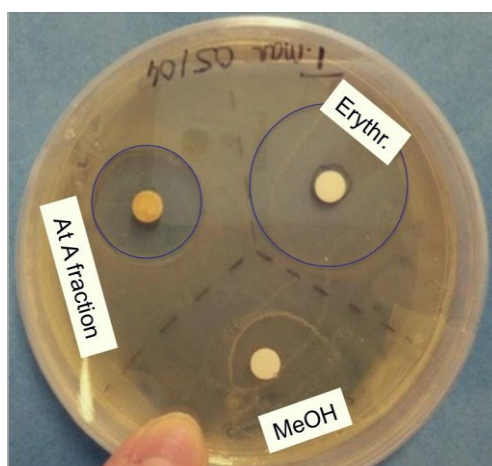


Figure 2. Disk assays tests of the *A. taxiformis* (At) fraction A. Inhibition zones are delimited with blue circles.

3.3. HPLC coupled antibacterial assays

Complementary antibacterial assays coupled to HPLC separation were pursued with the A fraction of *A. taxifomis* extract (MeOH) (2014 and 2015) to localise bioactive regions of the chromatogram. For each replicate, 100 % of inhibition of *T. maritimum* growth was observed for the 75th well (Figure 3). We analysed antibacterial bioactivity and chemical profile of *A. taxiformis* extracts from different years and months (2014, 2015 and 2016) and they all displayed strong antibacterial activities and the same chemical profile (Figure 3). Purification and characterisation of the antibacterial compound or compounds corresponding to the 75th well from the methanolic fraction of *A. taxiformis* (2015, 2016) is being currently performed at the laboratory.

4. Discussion

V. harveyi and *T. maritimum* are both serious disease agents in aquaculture, causing heavy losses worldwide (Brian and Zhang, 2006; Avendaño-Herrera et al. 2006b). Repetitive antimicrobial treatment has resulted in the development of *V. harveyi* and *T. maritimum* resistant strains (Avendaño-Herrera et al. 2007; Stalin and Srinivasan, 2016). Therefore, there is an urgent need to develop sustainable biocontrol strategies. In this study we have evaluated the antibacterial capacity of several Polynesian plants and algae against *T. maritimum* and *V. harveyi* strains from Tahiti.

Garlic (Amaryllidaceae) and turmeric (Zingiberaceae) presented strong antibacterial activities against *V. harveyi* at 2 mg/mL; however, they did not present antibacterial activity against *T. maritimum* at the concentration tested (500 µg per disk). Garlic antibacterial properties have been widely studied on marine pathogenic bacteria, including on multidrug resistant *V. harveyi* strains (Vaseeharan et al. 2011; Lee and Gao, 2012). Garlic multiple biological activities (e.g. antibacterial, antifungal, antiparasitic, antioxidant and immunostimulant) have mostly been associated to organosulfur compounds, particularly cysteine sulphoxides and thiosulphinates (Suleria et al. 2012). Allicin (diallylthiosulphinates), which is enzymatically formed from its precursor alliin when garlic is crushed, is considered

the most bioactive compound of garlic, and its antibacterial activities have been confirmed against a large number of human and fish pathogenic bacteria (Ankri and Mirelman, 1999; Nya et al. 2010). However, allicin is a labile compound easily transformed to a number of stable lipid-soluble allylsul des such as ajoene, which is also reported to have antibacterial activity (Naganawa et al. 1996; Viswanathan et al. 2014). Turmeric is also known to display numerous bioactivities, with reported antibacterial activities against a wide range of human and animal pathogenic bacteria, including *V. harveyi* and methicillin resistant *Staphylococcus aureus* (Kim et al. 2005; Lawhavinit et al. 2010). Curcuminoids, which include mainly curcumin, demethoxycurcumin and bisdemethoxycurcumin, are the most bioactive compounds of turmeric (Balaji and Chempakam, 2010).

A. taxiformis was the only algae to display antibacterial activity, and the only extract to display activity against both of the bacteria tested, *V. harveyi* and *T. maritimum*. *A. taxiformis* extract has been reported to display potent antibacterial, antiparasitic and antifungal activities against human and marine pathogens (Hutson et al. 2012 ; Genovese et al. 2012, 2013; Vitale et al. 2015). *A. taxiformis* produces numerous low molecular weight halogenated compounds including methanes, ethenes, acetic acids, acetamides, propanols, propanones, propenes, acrylic acids, propylene oxide, propyl and propanyl acetates, butenols, butenones and the recently described cyclopentenones (mahorone and 5-bromomahorone), which exhibit an array of biological activities including antibacterial and cytotoxic activities (Burresson et al. 1976; Greff et al. 2014). In our study, we found only one compound (or a group of similar compounds being eluted simultaneously) responsible for the antibacterial activity observed, and its purification and characterization is being currently performed in our laboratory. The bioactive compound/compounds are eluted at the end of the chromatogram, therefore we think they might be more lypophilic compounds than the previously mahorones described by Greff et al. (2014).

A. taxiformis can also inhibit quorum sensing and the proposed compound responsible for this activity is a 2-dodecanoyloxyethanesulfonate ($C_{14}H_{27}O_5S$) (Jha et al. 2013). Both *V. harveyi* and *T. maritimum* are known to have quorum sensing (QS) mechanisms, therefore, *A. taxiformis* antibacterial activity observed against both bacteria could be related to inhibition of QS (Defoirdt et al. 2008; Romero et al. 2010).

We observed a potent antibacterial activity of *A. taxiformis* methanolic extract against the aquaculture pathogenic bacteria *V. harveyi* and *T. maritimum*, and the characterisation of the bioactive compounds is currently being performed at the laboratory. Algae display a promising potential to be used in aquaculture against pathogenic infections due to their varied bioactive components. However, in order to evaluate their true potential in fish and shellfish aquaculture, *in vivo* experiences must be done to determine their effects on animal's physiology. Some studies have reported *A. taxiformis* immunostimulant activities in shrimp (*Penaeus monodon*), however, to date no study has yet evaluated the effect of *A. taxiformis* on fish immune system (Manilal et al. 2012, 2013). A recent study showed *A. taxiformis* aqueous extract was highly toxic to fish when they were immersed in it (Mata et al. 2013). Therefore, there is an urgent need to evaluate algae antibacterial activities together with *in vivo* fish treatments in order to evaluate the algae true potential as treatment of bacterial infections in aquaculture.



CHAPTER IX

Effects of local Polynesian plants and algae on growth and expression of two immune-related genes in orbicular batfish (*Platax orbicularis*)

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Effects of local Polynesian plants and algae on growth and expression of two immune-related genes in orbicular batfish (*Platax orbicularis*)

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Abstract

The emerging orbicular batfish (*Platax orbicularis*) aquaculture is the most important fish aquaculture industry in French Polynesia. However, bacterial infections are causing severe mortality episodes. Therefore, there is an urgent need to find an effective management solution. Besides the supplying difficulty and high costs of veterinary drugs in French Polynesia, batfish aquaculture takes place close to the coral reef, where use of synthetic persistent drugs should be restricted. Medicinal plants and bioactive algae are emerging as a cheaper and more sustainable alternative to chemical drugs. We have studied the effect of local Polynesian plants and the local opportunistic algae *Asparagopsis taxiformis* on batfish when orally administered. Weight gain and expression of two immune-related genes (lysozyme g – Lys G and transforming growth factor beta - TGF-β1) were studied to analyze immunostimulant activity of plants on *P. orbicularis*. Results showed that several plants increased Lys G and TGF-β1 expression on orbicular batfish after 2 and 3 weeks of oral administration. *A. taxiformis* was the plant displaying the most promising results, promoting a weight gain of 24% after 3 weeks of oral administration and significantly increasing the relative amount of both Lys G and TGF-β1 transcripts in kidney and spleen of *P. orbicularis*.

Keywords: orbicular batfish, gene-expression, lysozyme, transforming-growth factor, *Asparagopsis taxiformis*.

Highlights

- Different plants and algae were orally administered to orbicular batfish.
- Immune-related gene primers were for the first time identified in *Platax orbicularis*.
- Several plants increased the relative expression levels of Lys G and TGF- β 1 genes.
- *Asparagopsis taxiformis* induced a weight gain in *P. orbicularis* fingerlings.
- *A. taxiformis* increased immune-related genes expression in *P. orbicularis*.

1. Introduction

World aquaculture production keeps steadily growing, reaching 70.2 million tons of farmed fish in 2013, an increase of 5.6% from 2012 (FAO, 2014). However, aquaculture growth is often linked to culture intensification, which leads to overcrowding and poor water quality and facilitates the spread of pathogens and disease outbreaks (Bondad-Reantaso et al. 2005). Several drugs, like antibiotic and anthelmintic drugs, are commonly used by fish farmers to prevent and treat disease outbreaks (Rico et al. 2013). However, the intensive use of drugs presents numerous disadvantages and can cause 1) accumulation in the muscle of commercialized animals, 2) development of resistance and 3) undesirable effects on the local environment (e.g. Cabello, 2006; Marshall and Levy, 2013). Besides, the rising cost of prescription drugs also limits their application in many developing countries where aquaculture is one of the main supplies of animal protein (Hoareau and DaSilva, 1999). Medicinal plants appear to be a more sustainable and accessible alternative to synthetic drugs, and can at the same time boost fish fitness and immunity and help in pathogen prevention and treatment (Reverter et al. 2014). Medicinal plants have been reported to promote weight gain and enhance immunity in fish and shellfish as well as display antibacterial, antiviral, antifungal and antiparasitic activities against numerous aquaculture pathogens (Reverter et al. 2014). For example, oral administration of garlic powder (*Allium sativum*) promoted weight, enhanced immunity, showed antibacterial effects against *Aeromonas hydrophila* and *Vibrio harveyi* and antiparasitic effects against *Neobenedenia sp.* in several fish species (Sahu et al. 2007; Talpur and Ikhwanuddin, 2012; Militz et al. 2013). Turmeric (*Curcuma longa*) and ginger (*Zingiber officinalis*) also showed immunostimulant, antibacterial, antifungal, antiviral

and antiparasitic effects on fish and shellfish (Dügenci et al. 2003; Nya and Austin, 2009; Sahu et al. 2008). Some recent studies are also showing the promising potential of some algae in disease treatment and prevention in aquaculture (Dubber and Harder, 2008). Algae are considered to be a rich source of bioactive molecules and several *in vitro* studies have showed antibacterial, antiviral, antifungal and antiparasitic activities in different algae extracts (Choudhury et al. 2005; Hutson et al. 2012; Genovese et al. 2013). For example, the red algae *Asparagopsis taxiformis* displayed antibacterial, antifungal and antiparasitic activities against several fish pathogens and enhanced immune system of *Penaeus monodon* (Genovese et al. 2012, 2013; Manilal et al. 2013).

Orbicular batfish (*Platax orbicularis* - Forsskål, 1775, Ephippidae) live in brackish and marine waters around coral reefs, and is highly appreciated by the Polynesian and Chinese communities for its high quality meat and taste (Gasset and Remoissenet, 2011). *P. orbicularis* aquaculture is an emerging industry in French Polynesia, but advances in the control of its biological cycle together with the high economic value of this fish species, have contributed in its rapid development, mainly concentrated on Tahiti Island. However, orbicular batfish farming in Tahiti is suffering severe mortality episodes due to bacterial infections caused jointly by *V. harveyi* and *Tenacibaculum maritimum* shortly after the transfer of hatchery fingerlings to off-shore cages (D. Saulnier, pers. commun.). Since batfish aquaculture takes place close to coral reefs, synthetic drug utilization should be restricted and alternative treatments are required.

In the present study, we evaluate the capacity of some common medicinal Polynesian plants (garlic – *A. sativum*, turmeric – *C. longa*, ginger – *Z. marginalis* and noni – *Morinda taxifolia*) and algae (*A. taxiformis*) to increase expression of immune-related genes (lysozyme g and transforming growth factor) in orbicular batfish fingerlings when administered orally. Weight gain or loss of treated fish compared to control was also monitored to evaluate the effect of plants on fish appetite. Plants and algae were chosen according to reported bioactivities and local ethnobotanical knowledge (Sahu et al. 2007, 2008; Nayak and Mengi, 2009; Nya and Austin, 2009).

2. Materials and methods

2.1. *P. orbicularis* fingerlings and sampling

Healthy fingerlings of *P. orbicularis* were obtained from the Vaia hatchery located in Vairao (Tahiti, French Polynesia). Fish were placed in flow through 200 L tanks (35 fish per tank) with a water renewal of 100L/h and aeration via an airstone. Temperature, salinity and dissolved oxygen were measured daily and the unfed and fecal materials were removed once a day. Fish were fed 4 times a day with commercial food AL2G (Le Gouessant, Lamballe, France). Administered food quantities were precisely determined according to a feeding ratio based on standard production, which evolves with fish growth (5 to 10% of biomass depending on fish age, Gasset and Remoissenet, 2011). Experiments were carried out in triplicate tanks (3 tanks per treatment) and fish were sampled (3 fish/tank) after two or three weeks of treatment. Fish were weighted and spleen and kidney were collected aseptically and stored in RNA later (Ambion, Austin Texas, USA) at -80°C until RNA extraction (less than 2 months from collection time for most of the samples).

2.2. Diet

Fresh plant material (garlic –*A. sativum*, ginger –*Z. officinalis*, and turmeric –*C. longa*) was bought from local farmers, whereas noni (*M. citrifolia*) was collected in the Moorea rainforest (French Polynesia) and the red algae *A. taxiformis* was collected on the coral reef outer slope in Moorea. Noni and *A. taxiformis* as well as peeled garlic, ginger and turmeric bulbs were freeze-dried, powdered and stored at -20°C until used. Enriched diets were prepared adding 3% of sunflower oil, 3% of cod liver oil and the chosen proportion of the plant per kg of commercial fish food (Table 1). The plant enrichment proportion of 3% was chosen as a standard dose to evaluate efficacy differences between plants and then, two doses (3 and 1.5%) were selected for *A. taxiformis* to evaluate the dose effect on gene expression of immune-related genes. Control diet was also prepared with 3% of sunflower oil and 3% of cod liver oil to maintain the lipid content. One experiment without oil enrichment was also

performed to study the effect of oil on fish physiology and immunostimulant activity in particular.

Plant	2 weeks treatment % in fish food (w/w)	3 weeks treatment % in fish food (w/w)
<i>Morinda taxifolia</i>	-	3
<i>Zingiber officinalis</i>	-	3
<i>Allium sativum</i>	3	-
<i>Curcuma longa</i>	3	-
<i>Asparagopsis taxiformis</i>	3	3, 1.5

Table 1. Dietary plant enrichment specifications.

2.3. Immune-related gene expression study

2.3.1. Primer design

Since *P. orbicularis* is a non-model fish species, no sequences of immune-related or possible housekeeping genes were available on the GenBank database. Therefore, several primer sets were selected on the basis of a multiple alignment of nucleotide sequences of transforming growth factor (TGF- β 1), lysozyme G (Lys G) and alpha-actin genes (α -actin) from other fish species. Both lysozyme G and transforming growth factor genes were selected due to their key role in fish immune defense, and their potent regulatory activities on other immune molecules such as cytokines and complement (Li et al. 2006; Saurabh and Sahoo, 2008). These primers were designed using conserved regions of each gene and with Primer3 software to allow an optimal annealing temperature of 60°C +/- 2°C. Control or invariant internal genes were necessary for the global normalization of the quantification by real-time PCR (qPCR). The targeted candidate control genes were α -actin and elongation factor alpha (EF1 α), which were validated in several other fish species (Varsamos et al. 2006; Tang et al. 2007; Mo et al. 2014).

A total of 17 combinations of forward and reverse primers (4 μ M) for the 4 genes were analyzed by qPCR on dilutions of a reference cDNA sample obtained from a pool of cDNAs from spleen and kidney *P. orbicularis* tissues (see next section below). For each primer combination, seven series of dilutions tested in triplicate were used to establish the relationship between threshold cycle (Ct) qPCR values and log₁₀ of the reference cDNA

template. Couple of primers yielding both a qPCR efficiency ratio of almost 100% (restricted to a range between 90 and 110%), and the higher qPCR sensitivity, as expressed as the lowest cycle threshold (Ct) values for a given dilution, were selected. The specificity of the retained couple of primers was firstly checked by electrophoresis on a 1% agarose gel of qPCR products, using a DNA fluorescent dye and a DNA molecular weight marker and visualizing a single amplicon of the attempted size. Finally four amplicons of each targeted gene were purified on a QIAEX II gel extraction kit (Qiagen, Courtaboeuf, France), cloned on TOPO® TA cloning® kit (Invitrogen, CergyPontoise, France) and sequenced. For each targeted gene, one single sequence was obtained and deposited in GenBank database (Table 2) after verifying edited sequence by Basic Local Alignment Search Tool (BLAST).

Gene	Oligonucleotide sequences (5'–3')	qPCR efficiency (%)	Amplicon Length (bp)	Accession n°
EF1 α	GGCTGGTATCTCCAAGAACG GTCTCCAGCATGTTGTCTCC	106	239	KU950348
α -actin	GACTACCTCATGAAGATCCTGAC AGCTTCTCCTTGATGTCACG	102	89	KU976283
Lys G	GCTCTCATTGCTGCCATCAT TCAACCTGCATCAGTCCCA	98	100	KU976284
TGF- β 1	TCCCTCTACAACAGCACCAAG CAGGACCCCATGCAGTAGTT	93	758	KU950349

Table 2. Characteristics of the primers used to amplify the different genes.

cDNA synthesis and gene expression analysis.

RNA was isolated from *P. orbicularis* spleen and kidney tissues using the kit «SV Total RNA Isolation System» from Promega (Madison, WI, USA). Disruption of the cells was carried out by agitation with metal beads for 15 min (30 agitations/s) using a bead-beating device. Concentration of the nucleic acids was quantified by measuring the absorbance at 260 nm using a Thermo Scientific Nanodrop 1000 Spectrophotometer (Wilmington, Pennsylvania, USA). Purity of the samples was checked by measuring the ratio of OD 260/280 nm and 230/260 nm, and samples with a ratio lower than 1.8 or higher than 2.1 were purified again by isopropanol and ethanol precipitations. cDNA was generated using the «Transcriptor First

Strand - DNA Synthesis kit» from Roche (Roche Applied Science, Penzberg, Germany) and 500 ng of total RNA. Each cDNA sample amplification was performed in duplicate using a Mx3000P thermal cycler (Agilent Technologies, Santa Clara, California, USA). Each reaction contained 12.5 μ L of Brilliant II SYBR Green qPCR Master Mix (Agilent Tech.), 4 μ M forward and reverse primer and 10 μ L of template formerly diluted at 1:100 in pure water, in a final reaction volume of 25 μ l. The cycling conditions were 10 min at 95°C to allow the enzyme activation followed by 40 cycles (denaturation 30 s at 95°C, annealing 1 min at 60°C and 30 s extension at 72°C) and 1 min at 95°C and finally increasing temperature from 45°C to 95°C to obtain the melting curves. Threshold Cycle (C_t) value corresponded to the PCR cycle number at which an increase in reported fluorescence above the baseline signals was first detected. The threshold was set using an amplification-based algorithm from the Mx3000 software (Agilent Technologies) for the initial plate. For the other plates an inter-plate calibrator was used to set the threshold manually and ensure the repeatability of measures.

The relative expression of TGF- β 1 and Lys G genes were calculated using the comparative C_t method also referred to as the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Briefly, each immune-related gene expression level in fresh plant material condition was normalized with two housekeeping genes (α -actin and EF1 α) ($\Delta C_t = C_{t_{\text{target gene}}} - C_{\text{mean housekeeping genes}}$) and compared to the control diet condition ($\Delta\Delta C_t = \Delta C_{t_{\text{target gene}}} - \Delta C_{t_{\text{control}}}$) to yield relative immune-related gene expression rate ($2^{-\Delta\Delta C_t}$).

2.4 Statistical analysis

All experimental tests were performed by triplicate and mean \pm S.D. was calculated. Gene expression results were displayed using boxplots (package ggplot2 for R), where the median and the first and third quartile were represented. Normality of data distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) were tested and not satisfied, thus non-parametric tests were used. Mann-Whitney U test was used to identify differences among treatments. Significance level was considered at $P < 0.05$.

3. Results

3.1. Growth

None of the enriched diets (plants and algae) displayed a negative effect on fish growth. Only the fish fed with a diet supplemented in *A. taxiformis* presented a significantly higher growth ($P < 0.05$) than those fed with the control diet. Fish fed for two weeks on an enriched diet in *A. taxiformis* (3%) presented a weight gain of 13.8%, while fish fed for 3 weeks in *A. taxiformis*

(1.5 and 3%) presented respectively 23.8% and 14.8% weight gain (Table 3).

Plant	Concentration (%)	Length of treatment	Control weight (mean \pm S.D.)	Treatment (mean \pm S.D.)	Weight gain (%)	P-value
Oil control	3 (vegetal) +3 (cod)	2	3.43 \pm 0.65	3.64 \pm 0.74	5.78	> 0.1
<i>A. sativum</i>	3	2	12.25 \pm 1.06	12.68 \pm 1.24	3.39	> 0.1
<i>C. longa</i>	3	2	12.25 \pm 1.06	12.28 \pm 1.05	0.24	> 0.1
<i>A. taxiformis</i>	3	2	3.63 \pm 0.73	4.13 \pm 0.57	13.77	< 0.05
<i>A. taxiformis</i>	3	3	4.95 \pm 0.8	6.13 \pm 1.17	23.84	< 0.05
<i>A. taxiformis</i>	1.5	3	4.95 \pm 0.8	5.68 \pm 1.1	14.75	0.1
<i>M. taxifolia</i>	3	3	12.45 \pm 1.98	12.94 \pm 2.76	3.78	> 0.1
<i>Z. officinale</i>	3	3	9.70 \pm 1.22	9.64 \pm 0.95	- 0.62	> 0.1

Table 3. Weight results after the different diet treatments of *P. orbicularis* fingerlings.

3.2. Immunomodulatory effect

Two immune-related genes were identified for the first time in *P. orbicularis*. The relative expression of genes encoding lysozyme g (Lys G) and transforming growth factor (TGF- β 1) were measured in spleen and kidney of *P. orbicularis*.

Since we used a control diet enriched in oil to maintain the same lipid content as our treatment diets, a first assay to test the oil effect on the expression of the studied immune-related genes was performed. Oil did not increase expression level of neither Lys G nor TGF- β 1 in *P. orbicularis* fingerlings. Fish fed with an enriched diet in turmeric had significant higher expression of Lys G in kidney ($P < 0.05$, Figure 1), while fish fed with garlic presented significant higher expression of Lys G in spleen ($P < 0.05$, Figure 1). Fish fed during 3 weeks with an enriched diet in *A. taxiformis* (3%) had significant higher expression of Lys G in both spleen and kidney and significant higher expression of TGF- β 1 in kidney ($P < 0.05$, Figure 1 and 2). Fish fed during 3 weeks with an enriched diet in *A. taxiformis* (1.5%) presented significant higher expression levels of Lys g and TGF- β 1 in kidney ($P < 0.05$, Figure 1 and 2). However, fish fed with *A. taxiformis* for 2 weeks did not display an increased expression level of Lys g or TGF- β 1 in any of the organs. Ginger treated fish displayed a moderately but significant higher expression level of TGF- β 1 in spleen. No immunostimulatory effect was observed with the noni-enriched diet. No immunosuppression effects were observed in any of the treated fish in our experiments.

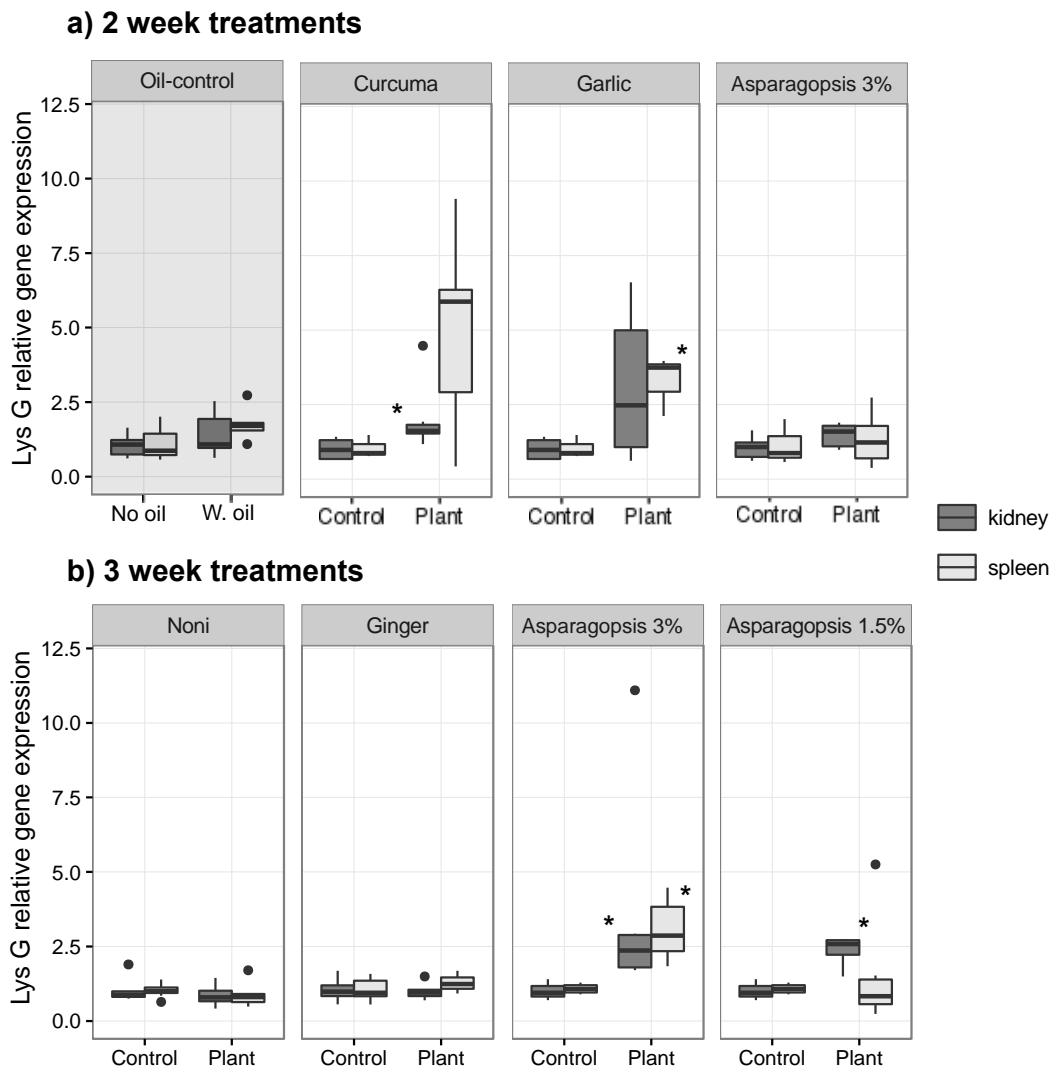


Figure 1. Lys G relative gene expression on kidney and spleen of *P. orbicularis* following 2 week treatment (a) and 3 week treatment (b) with diets enriched in several plants or algae. Oil enrichment control test is displayed in the grey rectangle. No-oil stands for commercial food alone, and W. oil represents commercial food enriched in oil (control used in the rest of the experiments). Upper hinge and lower hinge represent the first and third quartile, while upper whisker and lower whisker represent maximum and minimum values excluding the outliers (represented as black dots). * indicate P -value < 0.05 (Mann-Whitney U tests).

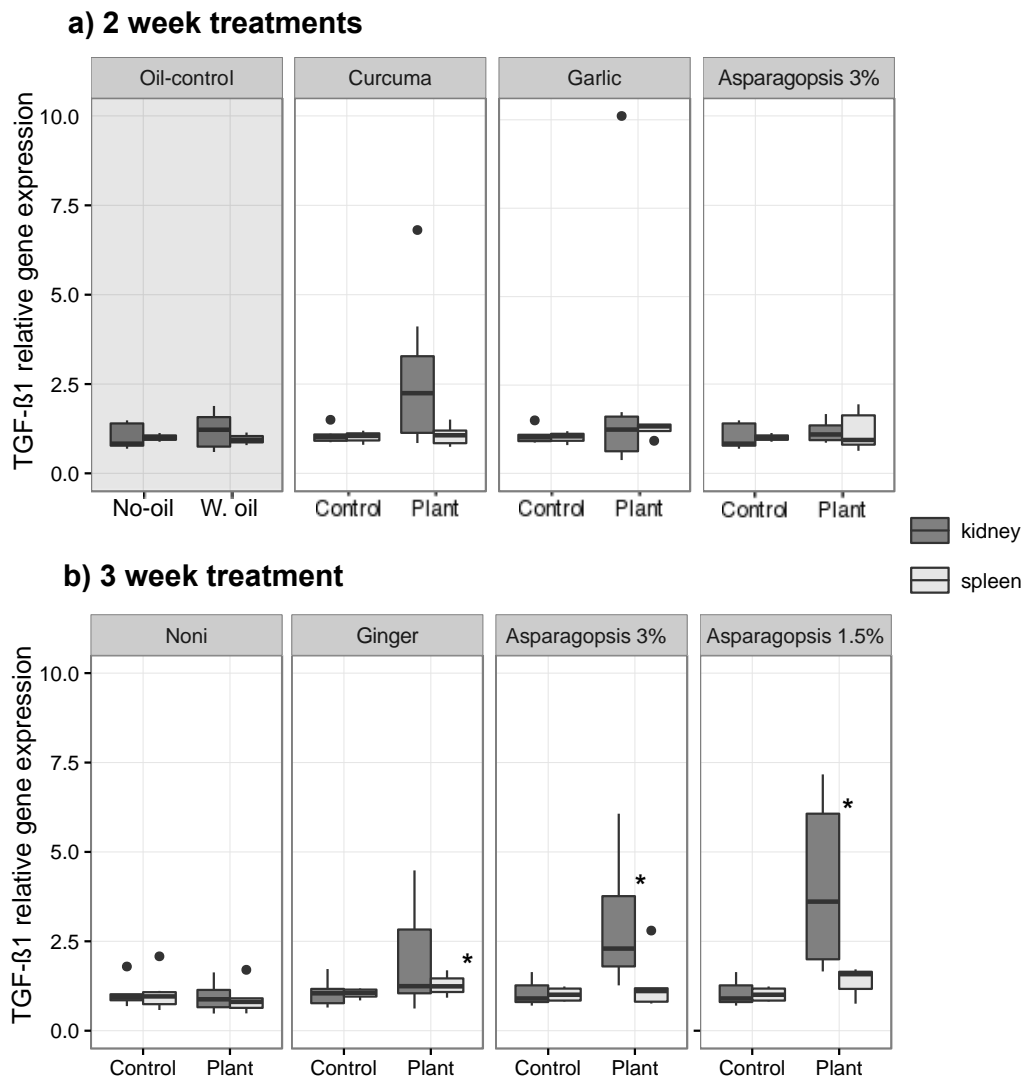


Figure 2. TGF- β 1 relative gene expression on kidney and spleen of *P. orbicularis* following 2 week treatment (a) and 3 week treatment (b) with diets enriched in several plants or. No-oil stands for commercial food alone, and W. oil represents commercial food enriched in oil (control used in the rest of the experiments). Upper hinge and lower hinge represent the first and third quartile, while upper whisker and lower whisker represent maximum and minimum values excluding the outliers (represented as black dots). * indicate P -value < 0.05 (Mann-Whitney U tests).

4. Discussion

An increasing number of studies report medicinal plant bioactivities in fish and against fish pathogens, making bioactive plants and algae a new alternative to prevent and treat disease outbreaks in aquaculture (Reverter et al. 2014). Our study has revealed the potential of several local Polynesian plants and algae to enhance the expression of two immune-related genes (Lys G and TGF- β 1) in orbicular batfish after 2 and/or 3 weeks of oral administration.

Lysozyme is a bacteriolytic enzyme that acts disrupting mucopolysaccharides in the bacterial cell walls, causing bacteria death (Chipman and Sharon, 1969). Lysozyme can also trigger other immune responses such as the complement system and phagocytic cells (Magnadottir, 2006). Therefore, lysozymes play an important role in the defense of fish and an increased level of lysozyme observed after dietary administration of garlic, turmeric and *A. taxiformis*, might improve fish performance against pathogenic infections. For example, Park and Choi (2012) showed that Nile tilapia (*Oreochromis niloticus*) fed with diets containing mistletoe (*Viscum album coloratum*) displayed increased levels of lysozyme and when challenged with the bacteria *Aeromonas hydrophila*, survivability of treated fish increased by 42%. Several studies have shown an increase of lysozyme and immunostimulant activity in other fish species after ginger, garlic and turmeric dietary administration (Sahu et al. 2008; Nya and Austin, 2009; Talpur and Ikhwanuddin, 2012). We did not observe an increased expression of lysozyme G after 3 weeks of ginger enriched diet administration. Plant chemodiversity varies between individuals of the same species depending on plant ontogeny, environmental and genetic factors (Moore et al. 2014). Variability in secondary metabolites and biological activities have been reported in ginger species, therefore we could think that the absence of immunostimulatory activity of ginger on *P. orbicularis* fingerlings could be related to the specific ginger chemotype used in this experiment (Ghasemzadeh et al. 2016). However, we can not exclude that maybe ginger dose used (3 %) or treatment length (3 weeks) could also not be the optimal to observe ginger immunostimulant effect on lysozyme g gene.

Transforming growth factor β is an immune regulator cytokine involved in wound repair processes, pro-inflammatory reactions and haematopoiesis (McCartney-Francis and Wahl, 1994; Lawrence, 1996). Atiba et al. (2011) showed that higher levels of TGF- β 1 due to *Aloe*

vera administration accelerated wound healing in rats. Other studies have shown that some immunostimulants and probiotics can increase levels of TGF- β 1 expression in fish (Panigrahi et al. 2007; Awad et al. 2011). In our study, TGF- β 1 expression increased significantly in the kidney of fish fed with an enriched diet in *A. taxiformis* for three weeks, and a moderate increase was also observed in the spleen and kidney of ginger treated fish.

Length of treatment and dose are also important parameters when assessing plant effects on fish physiology, since inappropriate doses can either be not effective or display toxic effects on fish (Kavitha et al. 2012; Militz et al. 2013). In our study, length played a notable effect on the immunostimulant activity of *A. taxiformis* in orbicular batfish, and a week-longer treatment caused a significant higher expression of two immune-related genes. However, we did not observe very different responses between diets with two *A. taxiformis* doses (1.5 and 3%).

The widely distributed and rapidly spreading red algae *A. taxiformis*, is known to produce a high diversity of halogenated metabolites with multiple bioactivities (McConnell and Fenical, 1977; Greff et al. 2014, Dijoux et al. 2014; Andreakis et al. 2016). Some chemical ecology studies have shown that *Asparagopsis* brominated metabolites are involved in the control of epiphytic bacterial communities and quorum sensing inhibition activities from MeOH extracts of *A. taxiformis* have been found (Paul et al. 2006a; Jha et al. 2013). Paul et al. (2006b) showed that halogenated natural products from the sister species *Asparagopsis armata* deterred herbivorous feeding. In aquaculture, several studies have shown antibacterial, antifungal and antiparasitic properties of *A. taxiformis* extracts against fish pathogens (Hutson et al. 2012; Genovese et al. 2012, 2013). However, an *in vivo* study showed high toxicity of *A. taxiformis* aqueous extract in barramundi (*Lates calcarifer*) (Mata et al. 2013).

This is the first *in vivo* study to show that *A. taxiformis* increased growth and expression level of immune-related genes in fish, when administered orally. In this study we fed *P. orbicularis* fingerlings during 2 and 3 weeks with a diet enriched in *A. taxiformis* and fish did not show any sign of deterrence due to the bioactive metabolites from the algae but rather an increased appetite and weight gain. Although we did not find any sign of algae toxicity in fish at any of the doses tested, longer *in vivo* studies should be done in order to evaluate algae toxicity on fish physiology after long exposures to the algae metabolites. Besides, studies on

several fish species would be beneficial to understand whether some fish species are more susceptible than others to *A. taxiformis* metabolites, or it is rather the exposure or administration procedure which affects fish susceptibility to the algae. Finally, since *A. taxiformis* proliferation is increasing in tropical areas like French Polynesia, its commercial use as fish food complement would not involve the introduction of exogenous molecules in the environment (and facility of culturing the algae) (Mantelatto et al. 2013).

5. Conclusions

This study showed the potential of some plants like garlic, turmeric and *A. taxiformis* to be integrated in fish diets to increase expression of immune-related genes. This is the first study, where *A. taxiformis* was orally administered to fish, and results show its capacity to induce weight gain and increase level of two immune-related genes in the new cultured fish species *P. orbicularis*. Fish fed with garlic, turmeric and *A. taxiformis* increased the level of Lys G in the spleen and/or kidney of *P. orbicularis* fingerlings. Fish fed with and enriched diet in *A. taxiformis* for 3 weeks also increased the level of TGF- β 1 and promoted weight gain in *P. orbicularis* fingerlings. These data provide interesting information on the effect of *A. taxiformis* on orbicular batfish immunity, and it shows the promising potential of this algae to be used as a fish complement to promote weight gain and enhance immunity, without the introduction of exogenous molecules into the environment. Further studies involving *in vivo* challenges with pathogens in fish fed with enriched diets in *A. taxiformis* will be needed in order to assess *A. taxiformis* true potential to prevent or treat diseases outbreaks in aquaculture.



CHAPTER X

Effects of local Polynesian plants and algae against *Tenacibaculum maritimum* infections in orbicular batfish (*Platax orbicularis*)

Effects of local Polynesian plants and algae against *Tenacibaculum maritimum* infections in orbicular batfish (*Platax orbicularis*)

Abstract

The emerging *Platax orbicularis* aquaculture in French Polynesia is threatened by severe mortality episodes of fingerlings due to tenacibaculosis outbreaks. Tenacibaculosis is often treated by antibiotic bath treatments, which are expensive and induce considerable amounts of stress to fish, heightening the disease severity. Since tenacibaculosis often appears after a stress shock, its management should be focused on techniques that maximize fish fitness. Medicinal plants are reported to increase fish fitness by stimulating their growth, enhancing immunity and reducing stress. In this study we have investigated the potential of local Polynesian plants (*Allium sativum*, *Calophyllum Inophyllum*, *Curcuma longa*, *Morinda taxifolia* and *Zingiber officinalis*) and algae (*Asparagopsis taxiformis*) against *T. maritimum* infections in *P. orbicularis* fingerlings. We have fed *P. orbicularis* fingerlings in enriched diets with the plants or algae for a period of two or three weeks. After the feeding period the fish were bath challenged with *T. maritimum* and cumulative mortality was recorded for a week. Although we encountered several technical problems, mainly on the repeatability of our experiments, the fish fed with an enriched diet in garlic had a significant higher survivability than control fish.

Keywords: *Tenacibaculum maritimum* challenge, medicinal plants, *Platax orbicularis*.

1. Introduction

The emerging *Platax orbicularis* aquaculture in French Polynesia is threatened by severe mortality episodes of fingerlings due to tenacibaculosis outbreaks (Saulnier, pers. comm.). Tenacibaculosis is an ulcerative disease caused by the Gram-negative bacteria *Tenacibaculum maritimum* (Flavobacteriaceae) that affects a large number of fish species and causes significant economic losses worldwide (Avendaño-Herrera et al. 2006b). Tenacibaculosis causes mainly lesions on the body surface of fish, such as ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes (Ostland et al. 1999; Avendaño-Herrera et al. 2006b). Tenacibaculosis outbreaks are mainly reported in cultured fish, following environmental or mechanical stress of fish, but a recent study reported low prevalences of tenacibaculosis in wild coral reef fish (Abd el-Galil and Hashem, 2012b). To the best of our knowledge, no published study has yet reported tenacibaculosis outbreaks in orbicular batfish.

Chemotherapy either by oral administration (e.g. antibiotics like enrofloxacin, florfenicol and oxytetracycline) or bath treatment (e.g. hydrogen peroxide) has been widely used to control tenacibaculosis, but variable results have been obtained (McVicar and White, 1979; Cepeda and Santos, 2002, Avendaño-Herrera et al. 2005; 2006b). Fish become anorexic immediately post-infection, and thus bath treatment seems to be more effective than oral administration of antibiotics (Soltani et al. 1996). However, bath treatments are expensive, have environmental impacts and induce considerable amounts of stress to fish, especially when applied to young fingerlings (Kierner and Black, 1997). Vaccines are also considered to control tenacibaculosis but there is currently only one vaccine commercially available to prevent turbot (*Scophthalmus maximus*) tenacibaculosis. However, isolated *T. maritimum* from different fish species is genetically variable, indicating that the vaccine developed for turbot may not be effective in preventing tenacibaculosis in other fish species (Avendaño-Herrera et al. 2004; Toranzo et al. 2004). Since aquaculture of orbicular batfish is still emerging, no studies evaluating the effectiveness of chemotherapy in orbicular batfish against bacterial infections such as tenacibaculosis are available.

Tenacibaculosis outbreaks occur often after the transfer of fish from hatchery to both inshore and offshore net cages, taking advantage of immunosuppressed or already abraded

fish (Wakabayashi et al. 1986). Therefore, tenacibaculosis management should focus on the use of preventive sustainable techniques that allow maximization of fish fitness to avoid opportunistic outbreaks. Medicinal plants have been reported to promote weight gain, reduce stress and enhance immunity in fish and shellfish as well as display antibacterial, antiviral, antifungal and antiparasitic activities against numerous aquaculture pathogens (Reverter et al. 2014). Therefore, the administration of medicinal plants as a complement of fish diet might boost fish immunity and decrease susceptibility to *T. maritimum* infections.

Six experimental infections were performed to *P. orbicularis* fingerlings fed with different enriched diets in plants (*Allium sativum*, *Calophyllum Inophyllum*, *Curcuma longa*, *Morinda taxifolia* and *Zingiber officinalis*) and algae (*Asparagopsis taxiformis*) in order to test efficacy of diet enrichment against *T. maritimum* infection. Three experiments were performed at the CRIOBE laboratory facilities in Moorea (French Polynesia) and the other three were performed at the IFREMER Pacific Center in Vairao, Tahiti (French Polynesia) (Table 1).

2. Material and methods

2.1. *P. orbicularis* fingerlings

Healthy fingerlings of *P. orbicularis* were obtained from the Vaia hatchery located in Vairao (Tahiti, French Polynesia). Fish were stocked in flow through 200 L tanks (35 fish per tank) with a water renewal of 20L/h and aeration via an airstone. Temperature, salinity and dissolved oxygen were measured daily and the unfed and fecal materials were removed once a day. Fish were fed 4 times a day with commercial food AL2G (LeGouessant, Lamballe, France), according to a feeding ratio based on standard production (5 to 10% of biomass, Gasset É. and Remoissenet G., 2011). Experiments were carried out in triplicate (3 tanks per treatment).

2.2. Diet

Fresh plant material (garlic – *Allium sativum*, ginger – *Zingiber officinalis*, curcuma – *Curcuma longa* and tamanu oil – *Calophyllum inophyllum*) was bought from the local market, whereas noni (*Morinda citrifolia*) was collected in the rainforest and the red algae (*Asparagopsis taxiformis*) was collected in the outer slope of Moorea (French Polynesia).

Noni, *Asparagopsis* as well as peeled garlic, ginger and curcuma bulbs were freeze-dried, powdered and stored at -20°C until used. Enriched diets were prepared adding 3% of sunflower oil, 3% of cod liver oil and the chosen proportion of the plant per kg of commercial fish food. The enriched diet with tamanu was prepared substituting the sunflower oil by tamanu oil (Table 1). The plant enrichment proportion of 3% was chosen as a standard dose to evaluate efficacy differences between plants and then, two doses (3 and 1.5%) were selected for *A. taxiformis* to evaluate the dose effect. Control diet was also prepared with 3% of sunflower oil and 3% of cod liver oil to maintain the lipid content. One experiment without oil enrichment was also performed to study the effect of oil on fish physiology and disease resistance.

Plant	Site of experiment	2 weeks treatment % in fish food (w/w)	3 weeks treatment % in fish food (w/w)
<i>Calophyllum inophyllum</i>	Moorea	-	3
<i>Morinda taxifolia</i>	Moorea	-	3
<i>Zingiber officinalis</i>	Moorea	-	3
<i>Allium sativum</i>	Moorea / Tahiti	3	3
<i>Curcuma longa</i>	Tahiti	3	-
<i>Asparagopsis taxiformis</i>	Tahiti	3	3, 1.5

Table 1. Dietary plant enrichment specifications.

2.3. *Tenacibaculum maritimum* bath challenge

The fish were initially divided in two groups (6 tanks per group): enriched diet and control diet. On the 15th or 21st day of the experiment, fish were not fed for 24 hours, and then 3 tanks of each group were bath challenged in seawater with the bacteria *T. maritimum*.

Fish were transferred to 40 L aerated tanks, where 10 mL of *T. Maritimum* sowed liquid culture were added. *T. maritimum* (TFA4) was cultured in both solid and liquid cultures. Solid cultures were grown using Zobell Marine Agar (Difco™) and incubated at 24h at 27°C, while liquid culture of *T. maritimum* was grown in Zobell Marine Broth (Difco™) for 24-48 hours at 27°C under agitation. *T. Maritimum* sowed solution was prepared immediately before the challenge and was obtained using three solid *T. maritimum* cultures to sow the liquid culture. After 2 hours of exposure, fish were rinsed with seawater and returned to the 200 L tanks. Control fish (non challenged) were also placed in 40 L tanks for two hours with seawater in order to have the same stress level than challenged fish. Fish were then fed with their corresponding diets until the end of the experiment (7 days after infection). Dead fish were removed every two hours, and mortality was monitored.

2.4. Data analysis

Cumulative fish mortality (%) after 7 days post-infection was calculated for each treatment and control experiments and was expressed as the mean \pm standard deviation of the three replicate tanks. T-tests were performed to identify significant different mortalities (p-value < 0.5) between treated and control fish.

3. Results and discussion

Experimental infection of *P. orbicularis* with *T. maritimum* through bath challenge proved to be poorly reproducible. Mortality rates varied from 0% to 64% in control fish depending on the experiments, which greatly complicated the interpretation of the results concerning the plant treatments. Besides, in some experiments, highly different infection rates were observed among replicates of a same treatment.

If infection was successful, fish condition degraded very fast, and within few hours fish started to present visible signs of infection like damaged fins, tail-rot and white spots and ulcers on the body. Mortality started approximately 24 hours post-infection and stabilized between the 3rd and 5th day. Fish heavily infected presented a considerable loss of colour in spleen and other organs (Figure 1).

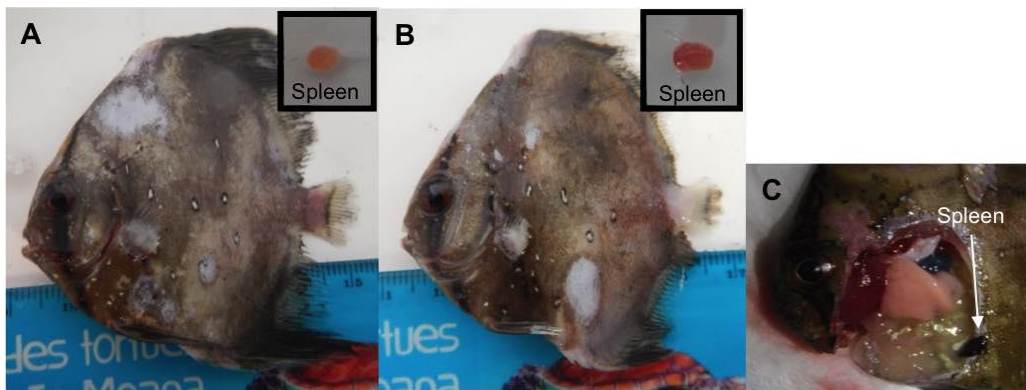


Figure 1. (A) and (B) Heavily infected *P. orbicularis* fingerlings with *T. maritimum* and their spleens. (C) Lightly infected *P. orbicularis* fingerlings and the normal spleen.

During the tamanu experiment, bad meteorological conditions contributed to specific culture conditions, with mean salinities of 18 ‰ and a high turbidity in the seawater. Although, the fish did not show any sign of stress and their growth was normal, the infection was not successful. *T. Maritimum* is a virulent bacteria, that causes big economic losses in the aquaculture industry, however it appears to be sensible to salinity, and some studies have shown that increased prevalences and severity of tenacibaculosis were observed at higher salinities (30 to 35 ‰) (Avendaño-Herrera et al. 2006b). To the best of our knowledge, no study has yet assessed turbidity effect on *T. maritimum* virulence and pathogenicity. *T. maritimum* is a highly filamentous bacteria, so it could be possible that high levels of suspended materials in the water could aggregate the bacteria, diminish available pathogenic bacteria to infect the fish, or decrease *T. maritimum* fitness, and thus decrease bacterial virulence. In fact, *P. orbicularis* fingerlings in the wild are found to inhabit shallow turbid waters where they drift around floating materials such as logs and ropes and mimic dead leaves. This habitat selection could result from a natural adaptation of the fish juveniles to protect themselves against pathogens.

The experimental infection in fish from the noni experiment was also unsuccessful. Fish did not show any behavioural change post-infection such as disorientation or appetite loss, and no signs of tenacibaculosis could be observed on their bodies or fins. Fish from the noni experiment weighted around 30 g, whereas fish in the other experiments weighted between 5 and 10 g. Different susceptibilities to *T. maritimum* associated with fish body weight have been observed before, however studies showed that *T. maritimum* resistance was observed in fish above 100 g (Avendaño-Herrera et al. 2005; 2006b). Therefore, higher fish weight could be a plausible hypothesis for the non-infection of *P. orbicularis* fingerlings during the noni experiment, but we cannot reject other explanations.

The third and last experiment in Moorea was on fish fed with an enriched diet of ginger and an enriched diet of garlic. Fish from these experiments were successfully infected, although infection was not even among replicates. Only two out of three control replicates were infected. The control mortality of the infected tanks was 12.86 ± 1.24 %, the ginger treated fish mortality was 27.61 ± 4.36 % and the garlic fed fish mortality was 6.67 ± 4.36 % (Figure 2A). Although, there was a poor repeatability among the control replicates, garlic treated fish had a significant higher survivability than the control fish and the ginger treated fish.

A second experiment with an enriched diet of garlic was performed, along with an enriched diet in curcuma. Despite low cumulative mortalities, (control = 5.37 ± 1.65 %, garlic = 1.07 ± 1.65 % and curcuma = 2.15 ± 1.65 %), garlic and curcuma treated fish displayed significantly (p -value < 0.05) lower mortalities than control fish (Figure 2B).

Finally, two experiments were performed on fish fed with an enriched diet in the red algae *A. taxiformis*, with different treatment lengths (2 and 3 weeks) and two doses (1,5 and 3 % of *A. taxiformis*). In the first experiment (2 weeks), a control diet without oil enrichment was also performed in order to test if the presence of high oil content in food played a role against *T. maritimum*. Results showed no significant differences in the fish mortality between the three treatments (control = 21.66 ± 6.24 , control without oil = 13.33 ± 6.24 , *A. taxiformis* = 18.33 ± 4.71 %). No significant differences in mortality were neither observed in the experiment where fish were fed for 3 weeks with an enriched diet of *A. taxiformis* 3% nor in fish fed with *A. taxiformis* 1.5 % (control = 64.70 ± 21.20 %, *A. taxiformis* 3% = 94.74 ± 9.12 %, *A. taxiformis* 1.5% = 82.35 ± 8.31 %) (Figure 2C and 2D).

Differences of *P. orbicularis* susceptibility between different experimental infections could be due to several factors. First of all, although all experimental infections are done following the same procedure, total *T. maritimum* concentration added to fish tanks during challenge might vary. A recent study has shown quorum sensing mechanisms (bacterial communication correlated to population density) in *T. maritimum* cultures (Romero et al. 2010). Bacterial cell communication and group behaviour provide significant benefits to bacteria in host colonization and biofilm formation, and most importantly, quorum sensing activities control expression of virulence factors of pathogenic bacteria (Li and Tian, 2012 ; Rutherford and Bassler 2012). Therefore, different concentrations of *T. maritimum* in experimental infections could play a major role in the virulence of infection. Different *P. orbicularis* fingerling susceptibilities to tenacibaculosis were also observed in the aquaculture farms, with different larvae productions, regardless of the period of the year. Our experiments, were separated in time, and were therefore performed on different larvae production, issued from different reproduction episodes. Since, *P. orbicularis* larvae production uses different breeders, we could think that some breeders could produce genetically more resistant *P. orbicularis* fingerlings to tenacibaculosis.

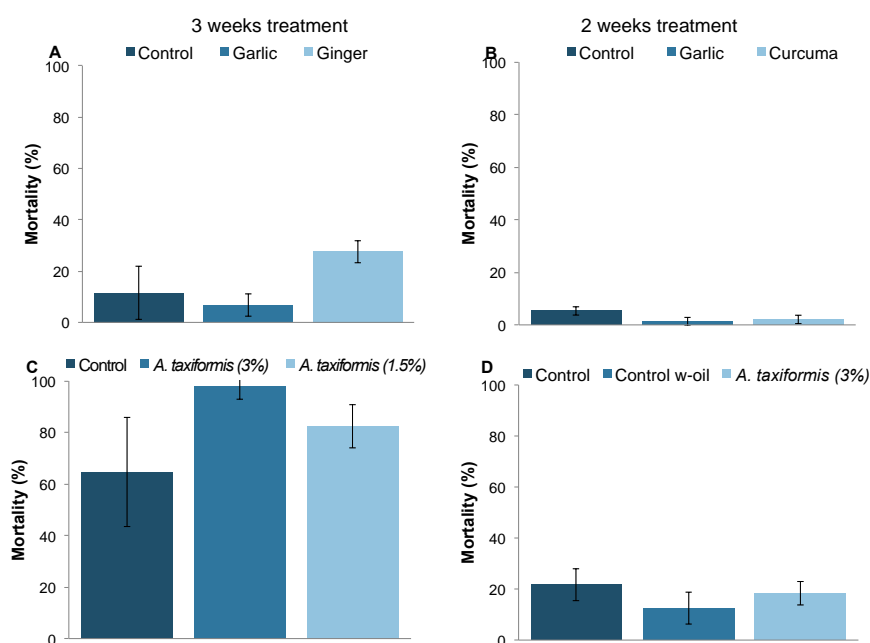


Figure 2. Mortalities (%) of *P. orbicularis* fingerlings infected with *T. maritimum* following different diet enrichments.

In summary, mortality levels varied a lot between the experiments, and different levels of infection could be observed between tanks of the same condition. Garlic seems to be the

most effective plant reducing mortality of *T. maritimum* infected *P. orbicularis* fingerlings. Mortality was reduced by 80% and 61% in fish fed with an enriched diet of garlic during 2 weeks and 3 weeks correspondingly. Fish fed with an enriched diet in curcuma also presented a reduction of mortality of 60%.

Conclusions and perspectives

Conclusions and perspectives

Parasites are an ubiquitous component of ecosystems, displaying important functional roles such as agents of natural selection and contributing to ecosystem organisation (Marcogliese, 2004; Poulin, 2014). Healthy natural ecosystems support a high parasite diversity, which contributes to the host-parasite balance and control of disease outbreaks (Johnson et al. 2013). However, under certain circumstances, such as climate perturbations or in high-density cultures, host-parasite interactions are modified, which can lead to outbreaks of infectious diseases (Hudson et al. 2006; Brooks and Hoberg, 2007; Pulkkinen et al. 2009).

Until now, chemotherapy has been widely used to prevent and treat disease outbreaks in culture systems such as aquaculture. Use of chemical drugs, however, presents multiple negative impacts on the environment and human health (e.g. development of bacterial strains and residual accumulation in animal tissue) (Reverter et al. 2014, Chapter VII). Besides, use of drugs such as antibiotics, has been shown to have immunosuppressive effects and alter the balance of the microbial community that provide protection against several infections, predisposing animals to repeated disease outbreaks (Rijkers et al. 1998; Sekirov et al. 2008). Therefore, disease management should concentrate on sustainable methods that increase fish fitness.

Medicinal plants have attracted increasing attention during the last few years as a potential alternative to chemotherapy in aquaculture. Plant products have been reported to stimulate appetite and promote weight gain, to act as immunostimulants and to have antibacterial and anti-parasitic (virus, protozoans, monogeneans) properties in fish and shellfish due to active molecules such as alkaloids, terpenoids, saponins and flavonoids (Reverter et al. 2014; Chapter VII). The emerging orbicular batfish (*P. orbicularis*) aquaculture in Tahiti is suffering severe mortality episodes due to bacterial infections (*T. maritimum* and *V. harveyi*) that compromise its development. In order to propose a local, cheap and sustainable treatment I have evaluated the potential of several local Polynesian plants and algae against *P. orbicularis* bacterial infections (Chapters VIII-X). The red opportunistic algae *A. taxiformis* displayed strong antibacterial activity against *V. harveyi* and *T. maritimum*, promoted weight gain and increased the expression level of two immune-related genes (Lys G and TGF- β 1) in orbicular batfish after three weeks of oral administration

(Chapters VIII, IX). Several studies have shown numerous biological activities of *A. taxiformis* extracts against fish pathogens, however, the only previous *in vivo* fish study showed high toxicity of *A. taxiformis* aqueous extract in farmed barramundi (*Lates calcarifer*) (Genovese et al. 2012, 2013; Mata et al. 2013). This is therefore, the first study in which oral administration of *A. taxiformis* in fish has been performed (Chapter IX). Results show a promising potential of *A. taxiformis* to be included in fish diets to increase fish fitness and enhance immune responses. However, further studies investigating the effectiveness of *A. taxiformis* enriched diet on disease protection in fish against bacterial pathogens need to be performed through *in vivo* challenge trials (Chapter X).

Better understanding of fish-parasite interactions and natural parasite evasion mechanisms can also provide new opportunities for sustainable parasite control in aquaculture. I have comprehensively studied the butterflyfish-monogenean interactions of 34 butterflyfish species from the tropical Indo-West Pacific Islands (Chapter I, II). Dactylogyrid parasites displayed high specificity, sympatric butterflyfish species presenting significantly different dactylogyrid communities (Chapter I). Results suggest that past biogeographical processes might have shaped parasite specificity probably through tight host-parasite coevolution. Spatial differences in the parasite nestedness (abundance gradient) were related to different environmental parameters, indicating the importance of favourable environmental conditions for dactylogyrid egg hatching and effective host infection (Chapter II). Recent studies have shown that environmental factors such as salinity or temperature are important factors in the arrangement of marine parasite communities (Thieltges et al. 2010; Poulin et al. 2011; Blasco-Costa et al. 2015). Higher temperature and salinity improved monogeneans egg hatching success, reduced time to sexual maturity and resulted in parasites reaching sexual maturity at a larger size (Tubbs et al. 2005; Lackenby et al. 2007; Brazenor and Hutson, 2015). The knowledge of environmental effects on parasite physiology are of high importance for an effective parasite management, and for example, Brazenor and Hutson recommended a more frequent treatment of fish stocks during warm temperature periods when parasites can complete their life cycle more quickly.

Out of the 34 butterflyfish species studied, I found only one species, *C. lunulatus*, that was never found parasitised by gill monogeneans (Chapter I). The ecology and phylogenetic position of *C. lunulatus* has been extensively studied (Pratchett, 2005; Pratchett et al. 2006; 2014, Fessler and Wesneat, 2007, Hsu et al. 2007) and there is no obvious peculiarity that

could explain this marked difference in parasitism. *C. lunulatus* is ecologically and phylogenetically close to species that are always heavily parasitized by gill monogeneans like *C. ornatissimus* and *C. reticulatus* (Hsu et al. 2007; Berumen et al. 2011). Besides, *C. lunulatus* is known to be parasitized by other gut and skin trematodes, indicating that gill monogenean absence must arise from gill or gill mucus species-specific characteristics.

I studied fish mucus because it is the first physical and biochemical barrier against pathogens (Chapter III). When *C. lunulatus* gill mucus was injected on the gills of three highly parasitized butterflyfishes, it induced a decrease in the number of gill monogenean parasites, indicating a possible antiparasitic activity of *C. lunulatus* gill mucus (Chapter VI). I have studied the gill mucus microbiome and metabolome of several butterflyfish species in order to investigate whether *C. lunulatus* had a specific microbiota and/or specific metabolites that could be related to the absence of gill monogenean parasites (Chapters IV, V). A microbiome study of four butterflyfish species (three highly parasitized species and *C. lunulatus*) revealed a high bacterial diversity in gill mucus of butterflyfishes, compared to previous studies on gill microbiome of cultured rainbow trout (*Oncorhynchus mykiss*) or gut microbiota of the coral reef surgeonfishes (Lowrey et al. 2015; Miyake et al. 2015) (Chapter IV). Microbiome of outer surfaces such as skin and gills has been reported to display a higher diversity than inner organs such as gut (Lowrey et al. 2015). Cultured fish microbiome is also expected to be less diverse than wild fish microbiome, which have higher genetic variability as well as higher diet plasticity (Givens et al. 2015). This study was the first to investigate wild fish gill mucus, therefore, more studies should be conducted to improve the knowledge on gill mucus microbiota. I found that a considerable proportion of butterflyfish gill mucus microbiota consisted of bacteria commonly found in the coral holobiont (Chapter IV). Since mucus layers are nutrient hotspots for marine heterotrophic bacteria living in oligotrophic environments such as coral reefs, I hypothesised that fish external mucus layers might act as a reservoir for coral reef bacterial diversity.

Metabolome study of eight butterflyfish species (four highly parasitized species, three low parasitized species and *C. lunulatus*) also revealed a high metabolic diversity, which differed significantly between fish species, fish with different diets, phylogenetic clade and parasitism level (Chapter V). Results showed that fish diet (obligate corallivore, facultative corallivore and omnivore) played a major role in the metabolite composition of gill mucus. Different food intakes can stimulate different physiological reactions in fish, which might

result in the differential production of metabolites in fish organs or tissues (Silva et al. 2014). Diet effect on metabolic profiles of fish internal organs such as liver are well reported, however, some studies have also reported expression of diet acquired metabolites such as mycosporine-like aminoacids (MAAs) or toxins in fish outer surfaces like skin mucus (Zamzow, 2007; Noguchi and Arakawa, 2008; Abro et al. 2014). Gill mucus metabolome of butterflyfishes was also influenced significantly by gill monogenean parasitism rates. Differences between parasitized and non-parasitized fish were the second most important factor explaining metabolic variability in the gill mucus polar fraction.

C. lunulatus gill mucus sustained a higher abundance of the anaerobic bacteria Fusobacteria and Spirochaetes that were positively correlated with overexpressed haemoglobin derived peptides in *C. lunulatus* gill mucus (Chapter IV). Fusobacteria is known to produce a short-chain fatty acid, butyrate, which enhances mucus production in mammals (Andoh et al. 1999). If Fusobacteria also enhances mucus production in fish, this would increase the gill mucus layer thickness, increasing oxygen diffusion distance and eventually promoting lower oxygen levels in gills of *C. lunulatus*. Haemoglobin expression has been reported to increase in several organisms under low oxygen levels, therefore, results suggest that *C. lunulatus* specific bacteria might play a role enhancing production of haemoglobin derived peptides (Chapter VI). In addition to its primordial function as oxygen carrier, haemoglobin is a known source of biologically active peptides that exhibit varied functions such as antimicrobial and anti-parasitic (Liepke et al. 2003; Ullal and Noga, 2010). Ullal et al. (2008) found that the expression of three β -chain haemoglobin peptides, one of which presented a high similarity with one of the *C. lunulatus* peptides, increased after ich (*Ichthyophthirium multifiliis*) infection in gill epithelium of channel catfish (*Ictalurus punctatus*). They found antimicrobial activities and strong anti-parasitic activities in one of the peptides, revealing their involvement in fish innate immunity against ectoparasitic infections (Ullal and Noga, 2010). Altogether, these results suggest that *C. lunulatus* native microbiota might play a role enhancing the production of haemoglobin derived peptides, most likely through the decrease of mucus oxygen levels, and therefore might protect this species from gill ectoparasites (Chapter VI). However, purification, synthesis and further evaluation of purified peptides biological activities will be performed in the laboratory, in order to confirm this hypothesis. It would be highly interesting to test whether an increased level of haemoglobin antimicrobial peptides would also occur in other Chaetodon species under lower

oxygen levels, or whether exclusive *C. lunulatus* bacteria (mainly Fusobacteria and Spirochaetes) play a more specific role in the production of haemoglobin peptides, for example by cleaving the haemoglobin protein as shown by Du et al. 2010. Experiments involving bacterial challenges of butterflyfishes and study of the haemoglobin peptides expression could cast light on the effect of bacteria in the production of butterflyfish haemoglobin peptides.

Antimicrobial peptides (AMPs) are normally small cationic peptides (usually less than 50 aa), which can either be derived from a biologically inactive proprotein that is processed to the active form or derived from larger, functional proteins such as histones or haemoglobins (Radek and Gallo, 2007). AMPs are one of the components of the non-specific immune system that offer a first line of protection in many animal species including fish (Falco et al. 2009). Besides having direct activity on a wide-range of microorganisms, AMPs modulate host innate and adaptive immunity by induction or modulation of pro-inflammatory cytokines and chemokines, apoptosis, gene transcription activities, inhibition of the inflammatory response, recruitment and stimulation of proliferation of macrophages, neutrophils, eosinophils and T lymphocytes, and the activation and differentiation of dendritic cells (Nijnik et al. 2009; Katzenback, 2015). AMPs can be produced at a constitutive level, however, the major pathway of AMP production is through the recognition of pathogen associated molecular patterns (PAMPs) via Toll-like receptors (TLRs) (Zasloff, 2002; Katzenback, 2015). AMPs provide promising applications in aquaculture and human health as an alternative to antibiotics, vaccine adjuvants and antitumor agents (Noga et al. 2011; Gautam et al. 2016). Jia et al. (2000) showed that continuous peritoneal deliverance of the antimicrobial peptides CEME (a cecropin-melittin hybrid peptide) and pleurocidin amide (a C-terminally amidated form of the natural flounder peptide) reduced mortality in *Vibrio anguillarum* infected coho salmon (*Onchorhynchus kisutch*). It has been claimed that bacteria are less likely to evolve resistance towards cationic antimicrobial peptides than conventional antibiotics (Zasloff, 2002). However, several studies showed that bacteria could evolve heritable mechanisms of resistance against cationic antimicrobial peptides, suggesting that, like conventional anti-infective agents, the therapeutic use of AMPs could result in the spread of resistant organisms (Perron et al. 2006; Sallum and Chen, 2008). Fish display a wide-variety of AMPs, therefore, instead of therapeutical use of targeted AMPs in aquaculture disease management, it would be preferential to trigger fish species-specific expression of

antimicrobial peptides (Masso-Silva et al. 2014). Casadei et al. (2013) found that several AMPs such as defensins, cathelicidins and liver AMPs were overexpressed after peptidoglycan administration in rainbow trout (*O. mykiss*). However, to date, little is still known on how induced AMP expression can be performed in cultured fish in order to prevent disease outbreaks. A recent study highlighted the use of probiotics to produce and secrete heterologous endogenous AMPs of interest in the human gastrointestinal tract (Mandal et al. 2014).

In the second part of this thesis, as discussed before, I have investigated the use of medicinal plants to fight against bacterial infections in orbicular batfish. It would be interesting to study the AMP composition of *P. orbicularis* external surfaces, which are the first surfaces to be attacked by *T. maritimum*, and their bioactivities. Once known which AMPs are naturally found in *P. orbicularis*, a sustainable method to control bacterial infections could focus on enhancing AMPs expression. *A. taxiformis* oral administration to orbicular batfish (*P. orbicularis*) increased the level of two immune-related genes (Lys G and TGF- β 1) (Chapter IX). However, the next step would be to test whether *A. taxiformis* can also induce a higher expression of antimicrobial peptides, and their effectivity against bacterial infections such as tenacibaculosis.

The comprehensive study of fish-parasite interactions can provide helpful insights in parasite evasion mechanisms of potential interest for aquaculture. Fish and their parasites have coevolved together for millions of years; therefore, natural systems can provide the best sustainable strategies against parasite outbreaks. Recent studies in humans and corals are showing that the complex interactions within one organism (the holobiont) play an important role in the organism's health, and disturbances within the complex community have drastic consequences for the well-being of the members (Clemente et al. 2012; Krediet et al. 2013). Aquaculture disease management is mostly focused on the use of chemical drugs that display direct effects against the targeted infectious organisms (Cabello et al. 2013). Cultured fish are subjected to regular stresses that deeply affect their fitness as well as their interactions with their commensal microbiota, which increase their susceptibility to pathogenic infections (Boutin et al. 2013). Therefore, disease management should concentrate on sustainable methods that increase fish fitness and re-establish their natural balance. In this thesis I have studied the potential effect of some plants and algae to enhance fish immunity and I have investigated the natural parasite evasion mechanisms of a species of butterflyfish, which

revealed the importance of the association between the commensal microbiota and the fish innate immune system. This study shows the importance to investigate host-parasite interactions under a global approach. The integration of epidemiology, biogeography, metabolomics and microbiomics show the complexity of fish-parasite relationships and the relevance of integrating different disciplines.

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ANNEXES

ANNEXE 1: Reverter, M., Bontemps, N., Sasal, P., Saulnier, D., 2017. Chapter 9. Use of medicinal plants in aquaculture. In: Austin, B., Newaj-Fyzul, A. Diagnosis and control of diseases of fish and shellfish. Wiley, Oxford. *In press.*

Chapter 9. Use of medicinal plants in aquaculture

Reverter M., Bontemps N., Sasal P., Saulnier, D.

1. Introduction

For thousands of years, civilisations throughout the world have used medicinal plants to treat a variety of diseases. The oldest written evidence of medicinal plants' usage was found on a Sumerian clay slab, approximately 5000 years old (Petrovska, 2012). Nowadays, traditional medicinal plants continue to be the primary source of healthcare in many developing countries and rural regions (Calixto, 2005). Ethnobotanical studies have proved to be highly useful in identifying bioactive plants, and numerous research works have been conducted on biological activities and chemical composition of ethnomedicinal plants (Banksota et al. 2003; Ayyanar et al. 2011). Interest in medicinal plants for human and veterinary health has been fuelled by the countless side effects, and rising costs of prescription drugs (Hoareau and Da-Silva, 1999). Plants also provide the advantage to possess a complex chemical composition which, 1) display varied biological activities, making plants suitable for the treatment of multifactorial diseases and 2) makes plants a suitable alternative to antibiotics with little risk for development of resistance (Srivastava et al. 2014; Gostner et al. 2012).

Aquaculture, is the fastest growing animal food producing sector, with an average annual increase of 6.2% per year in the period 2000-2012 (FAO, 2014). However, aquaculture growth is often linked to culture intensification, leading to overcrowding and poor water quality, facilitating the spread of pathogens and increasing disease outbreaks and mortality (Bondad-Reantaso et al. 2005). In order to avoid economic losses related to sanitary shortcomings, veterinary drugs are commonly used in aquaculture to prevent and treat disease outbreaks (Rico et al. 2013).

The intensive use of synthetic drugs, however presents numerous disadvantages, both for the environment and health safety. Intensive use of antibiotics have resulted in accumulation in muscle of commercialised animals (e.g. Cabello et al. 2006; Romero-Ormazabal et al. 2012) and the development of resistant bacteria strains (e.g. Miranda and Zemelman, 2002; Seyfried et al. 2010). Also, the use of antiparasitic drugs like trichlorfon or praziquantel in bath treatments is hazardous for animals and the environment and it can also result in the development of resistance (Umeda et al. 2006; Forwood et al. 2013). Vaccines, which are considered to be the most effective method to prevent disease outbreaks in aquaculture, are too expensive for widespread use by fish producers and, since it is extremely difficult to develop multiple strain vaccines, most vaccines are only effective against one type of pathogen (Sakai, 1999; Pasnik et al. 2005). Considering the numerous disadvantages of synthetic drugs, there is an increasing need for the development of alternative strategies in aquaculture disease management. Moreover, disease outbreaks in aquaculture are often associated with animal fitness and health, most pathogens being opportunistic and taking advantage of immunocompromised or stressed fish, thus alternative solutions should maximise fish immunity and fitness as a strategy to face pathogen infections

(Davis et al. 2002; Iguchi et al. 2003; Ashley et al. 2007). Medicinal plants, can therefore provide a cheaper and more sustainable alternative to chemotherapy in aquaculture, since they have been reported to display numerous bioactivities such as anti-stress, immunostimulant and anti-parasitic (bacterial, fungus, virus and ectoparasites) (Reverter et al. 2014).

2. Use of medicinal plants in aquaculture

Although interest in the use of medicinal plants and plant extracts in aquaculture has exploded recently, medicinal plants have long been used by some rural fish farmers. Caruso et al. (2013) found that 46% of the fish farmers surveyed in West Java (Indonesia) used plants in their farms, most of which were also traditionally used in human pharmacopeia. In most cases, fresh plants were directly introduced into the rearing water and used to 1) improve water quality, 2) reduce fish stress, 3) increase fish resistance to pathogens and 4) treat fish diseases. Their study showed that fish farmers used plants and doses depending on their personal experience and their ethnic origin.

Numerous scientific studies endorse the use of medicinal plants in aquaculture (e.g. Bhuvanewari and Balasundaram, 2006; Harikrishnan et al. 2011a). Plants have been reported to promote various activities like anti-stress, growth promotion, appetite stimulation, immunostimulation, aphrodisiac and to have anti-pathogen properties in fish and shrimp aquaculture due to highly varied active principles such as alkaloids, terpenoids, tannins, saponins or flavonoids (Citarasu, 2010; Chakraborty and Hancz, 2011).

2.1 Medicinal plants biological activities in aquaculture

Several plant extracts are reported to stimulate appetite and promote weight gain when they are administered to cultured fish (Pavaraj et al. 2011; Takaoka et al. 2011). For example, grouper *Epinephelus tauvina* fed with a supplemented diet of a mixture of methanolic herb extracts (*Cynodon dactylon*, *Piper long*, *Phyllanthus niruri*, *Tridax procumbens* and *Zingiber officinalis*) displayed 41% higher weight than fish fed with the control (Punitha et al. 2008). Some other studies have reported that administration of plant extracts improves digestibility and availability of nutrients, resulting in an increase in feed conversion and leading to higher protein synthesis (Putra et al. 2013; Talpur et al. 2013).

Numerous studies showed that fish treated with medicinal plants presented enhanced immune parameters (Düğenci et al. 2003; Yuan et al. 2007). For example, Nile tilapia (*Oreochromis niloticus*) fed with a diet containing mistletoe (*Viscum album coloratum*) for a period of 80 days displayed higher lysozyme activity, respiratory burst activity, alternative complement activity and phagocytic activity, which resulted in 42% of increased survivability when they were challenged with the bacterial pathogen *Aeromonas hydrophila* (Park and Choi, 2012). Studies on fish hematological parameters showed that fish treated with plants displayed higher levels of erythrocytes, lymphocytes, monocytes and hemoglobin than control fish, indicating better fish fitness (Harikrishnan et al. 2012a; Haghghi and Rohani, 2013).

In vitro and *in vivo* studies have showed the potential of medicinal plants against a wide range of marine pathogens (bacteria, virus, fungus and ectoparasites) (e.g. Direkbusarakom et al. 1996 ; Chitmanat et al. 2005; Ji et al. 2012). Antibacterial properties of medicinal plants have been by far the most studied biological activities, with abundant *in vitro* studies reporting antibacterial activity in numerous plants, against both gram-positive and gram-negative marine bacteria (e.g. Castro et al. 2008; Roomiani et al. 2013). *In vivo* studies in Indian major carp (*Labeo rohita*) fed with enriched diets in prickly chaff flower (*Achyranthes aspera*, 0,2%) and Indian ginseng (*Withania somnifera*, 0,5%) showed a reduction in mortality of 41% and 49% respectively when fish were challenged with *Aeromonas hydrophila* (Vasudeva-Rao et al. 2006; Sharma et al. 2010). Antiviral and antifungal activities of medicinal plants are equally interesting to prevent high mortality rates in aquaculture. Balasubramanian et al. (2008a, 2008b) showed that black tiger shrimp (*Penaeus monodon*) challenged with White Spot Syndrome Virus (WSSV) whilst treated with Bermuda grass (*Cynodon dactylon*) displayed no mortality and no signs of disease compared to 100% mortality observed in control groups. Several studies have shown *in vitro* antifungal activities of several plants like conidinium fruit (*Cnidium monnieri*), magnolia bark (*Magnolia officinalis*), aucklandia root (*Aucklandia lappa*) and common rue (*Ruta graveolens*) (Hashemi-Karouei et al. 2011; Xue-Gang et al. 2013). Medicinal plants seem to be also an effective alternative for treating ectoparasites. Several studies have shown antiparasitic activities of medicinal plants when added to water or administered orally (Yi et al. 2012; Huang et al. 2013; Fu et al. 2014). Methanol extract of bupleurum root (*Radix bupleuri chinensis*), aqueous and methanol extracts of cinnamon (*Cinnamomum cassia*), methanol extract of Chinese spic brush (*Lindera aggregata*) and methanol and ethyl acetate extracts of golden larch (*Pseudolarix kaempferi*) had 100% *in vivo* efficacy against monogenean *Dactylogyrus intermedium* in infected goldfish (*Carassius auratus*) (Wu et al. 2011; Ji et al. 2012). Harikrishnan et al. (2012c) showed 40% mortality decrease and enhanced immunity in olive flounder (*Paralichthys olivaceus*) infected by the protozoan ciliate *Miamiensis avidus* when fish were fed with a supplemented diet on *Suaeda maxima*.

2.2 Application of medicinal plants in aquaculture

Plants can be administered as a whole plant or parts (leaf, root, seed, fruit) and can either be used fresh or as prepared herbal extracts with different solvents (e.g. water, methanol, chloroform, ethyl acetate) (Van Hai, 2015). Biological activities and chemical composition of plants and extracts can vary greatly depending on the part used and type of extract, and thus previous knowledge of the plants' bioactive compounds is required. Besides, seasonal variations in production of bioactive metabolites has also been observed in different plants and algae, so a deep study on the plant production of the bioactive molecules is desirable (Manilal et al. 2009; Chaves et al. 2013).

To date, most scientific studies on the use of medicinal plants in aquaculture have focused on identification of biological activities rather than natural product determination (Balasubramanian et al. 2007; Cox et al. 2010; Ji et al. 2012). Exploratory studies on the potential of medicinal plants in

aquaculture can therefore be misleading if the wrong part or wrong solvent is used. Also, suitable dosing is crucial to obtain the desired effects, since inappropriate doses can display toxic effects on fish (Sambasivam et al. 2003; Ekanem et al. 2007; Kavitha et al. 2012). For example, grouper *Epinephelus coioides* fed with a supplemented diet of 1% of ethanol katuk extract (*Sauropus androgynous*) showed enhanced growth and improved food utilisation, whereas at 2.5 and 5% of katuk extract they presented lower growths (Putra et al. 2013). Treatment length is another important parameter in medicinal plant applications in aquaculture, since it influences directly the effectiveness of the treatment. For example, Militz et al. (2013) showed that farmed barramundii (*Lates calcarifer*) fed with an enriched diet of garlic (*Allium sativum*) for 30 days displayed 70% decreased *Neobenedenia sp.* infection success compared to control and short-term treatment (10 days).

Medicinal plants can be administered to fish and shellfish by injection (intramuscular and intraperitoneal), oral administration and through immersion or baths (Wu et al. 2010; Ji et al. 2012; Putra et al. 2013). Although intraperitoneal injection has proved to be the most rapid and efficient way of administration, it is expensive, laborious and stressful for fish, special for very young specimens (Anderson, 1992; Yoshida et al. 1995). Bath treatment is an extensively used technique for the treatment of ectoparasites (Whittington, 2012; Forwood et al. 2013). However, this method is also expensive and laborious and involves the release of exogenous molecules in the marine environment, and thus can present some undesired environmental side effects (Umeda et al. 2006). Hence, oral administration seems to be the most suitable for aquaculture, since medicinal plants can induce physiological changes in fish to improve their fitness and reinforce their resistance to pathogens.

Finally, determining plant natural products helps to better understand their mode of action. For example, chelerythrine and chelidonine are two benzo[c]phenanthridines with antimicrobial, antifungal and anti-inflammatory activities isolated from greater celandine (*Chelidonium majus* L.) (Waltérova et al. 1995; Malikova et al. 2006). Several studies have shown that chelerythrine exhibits high cytotoxic potency against a large number of cells, through multiple apoptosis-inducing pathways, indicating that a direct action on mitochondria may be involved in the eradication of parasites (Slaninová et al. 2001; Kemeny-Beke et al. 2006; Kaminsky et al. 2008). Other studies showed the mechanisms of action of chelidonine against the monogenean ectoparasite *Dactylogyrus intermedius* may be through mitochondrial cell death pathway activated through caspase-9 (Philchenkov et al. 2008). Therefore, it is of high importance to identify the bioactive molecules for the observed bioactivities and standardize extraction procedures depending on the bioactive molecules identified, application procedures as well as doses and duration of treatments depending on the type of pathogen (Reverter et al. 2014).

It is important to state that modes of action and toxicity of many plants are still poorly understood, so more research is needed before they can be safely used in aquaculture. Many of the plant bioactive molecules can also be toxic or antinutritional in fish, decreasing efficient utilisation of feed nutrients or causing intestinal dysfunction, so their effect on fish physiology and adequate dosing needs to be studied (Krogdahl et al. 2010). In order to fill in the gaps in current knowledge, a structured research plan should

be followed, focused on bringing light firstly on bioactivities, compounds and modes of actions of the plants, and secondly on the plant effect on organism physiology and its appropriate use. In figure 1 we propose some directions for research on medicinal plants utilisation in aquaculture. *In vitro* tests should be performed in order to determine plant bioactivities, together with bio-assay guided fractionation to identify and characterise the bioactive natural products. *In vitro* tests should also be carried to test cytotoxicity of plant extracts on fish cells, in order to have a first idea of the plant toxicity on the animals. Then *in vivo* tests on model species should be performed, firstly to understand the plant effects on animal physiology and determine the best conditions for plant application (mode of application, plant part and preparation, dose and length of treatment), and secondly to estimate plant efficiency in organism survival against different pathogenic infections. Hematology indicators such as hematocrit, hemoglobin, erythrocyte and lymphocyte levels can be used to determine fish fitness (Haghighi and Rohani, 2013; Kanani et al. 2014). Fish immunity is often analysed to see immunomodulatory power of plants and it can be either studied by classical biochemical approaches (e.g. lysozyme activity, phagocytic activity, respiratory burst activity) or by the study of immune gene expression (e.g. Lys, TNF- α , IL-1, IL-10 genes) (Harikrishnan et al. 2011e; Kumar et al. 2013; Chakrabarti et al. 2014).

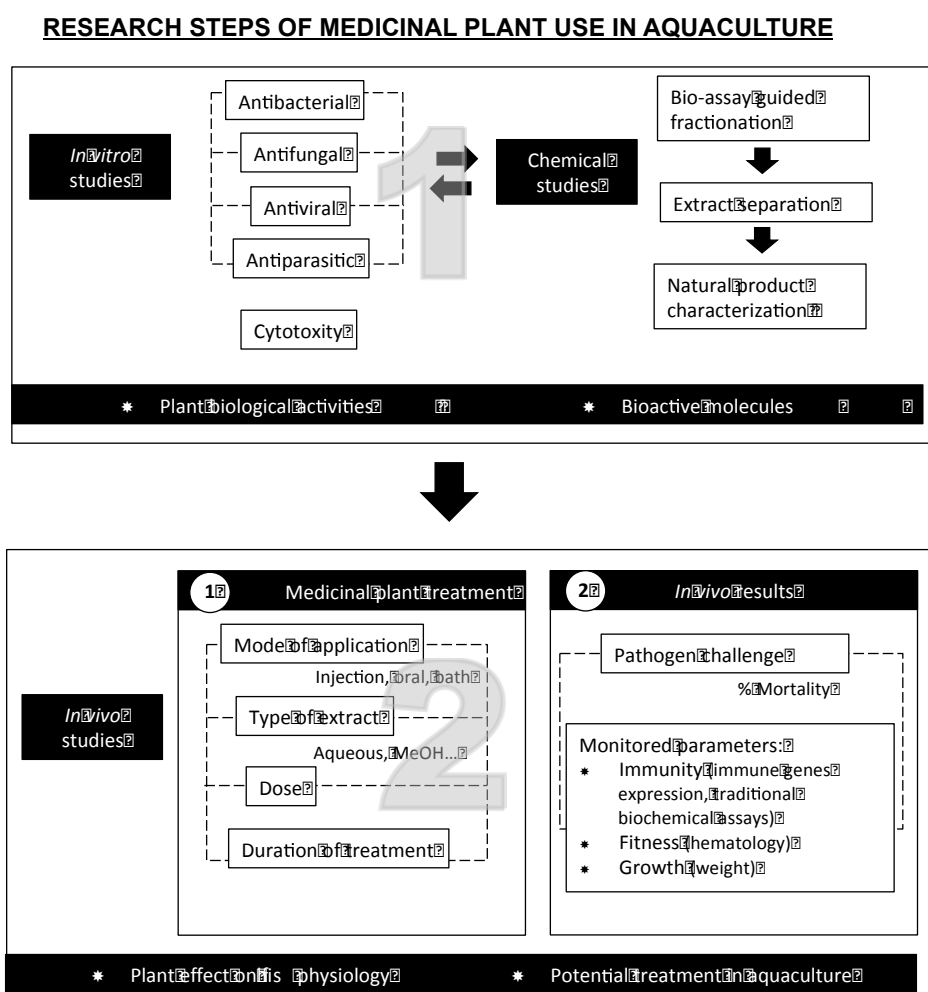


Figure 1. Research steps of medicinal plant utilisation in aquaculture.

3. Analysis of plants used in aquaculture

An extensive literature review was performed on the published studies on plant use in aquaculture in order to identify the most common studied plants and their activities (Table 1). Most of the plants used both by fish farmers and in scientific studies for the potential use in aquaculture correspond to medicinal plants widely used in traditional medicine. We identified over 250 plant species from 75 families and 32 orders, which have been used or reported as potentially interesting in aquaculture.

Order	Family	Species	Activities	Used part	Bigliography
Acorales	Acoraceae	<i>Acorus calamus</i>	AV	R	Sivasankar et al. 2015
Alismatales	Araceae	<i>Alocasia macrorrhizos</i>	NA	L	Caruso et al. 2013
Alismatales	Araceae	<i>Colocasia esculenta</i>	AB	R	Wet et al. 2010
Apiales	Apiaceae	<i>Angelica membranaceus</i>	IM	R	Jian and Wu, 2004
Apiales	Apiaceae	<i>Angelica sinensis</i>	IM	R	Wang et al. 2011
Apiales	Apiaceae	<i>Angelica pubescens</i>	AF	R	Xue-Gang et al. 2013
Apiales	Apiaceae	<i>Bupleurum chinense</i>	AP	W	Wu et al. 2011
Apiales	Apiaceae	<i>Centella asiatica</i>	AB	R	Rattanachaikunsopon and Phumkhachorn, 2010b
Apiales	Apiaceae	<i>Cnidium officinale</i>	AB,GP	L, Fr	Takaoka et al. 2007b
Apiales	Apiaceae	<i>Corandrium sativum</i>	AB	NA	Innocent et al. 2011
Apiales	Apiaceae	<i>Heracleum lasiopetalum</i>	AB	R	Pirbalouti et al. 2011
Apiales	Apiaceae	<i>Radix peucedani</i>	AP	W	Wu et al. 2011
Apiales	Araliaceae	<i>Kalopanax pictus</i>	AP, IM	S	Harikrishnan et al. 2011e
Apiales	Araliaceae	<i>Panax ginseng</i>	IM	R	Gooda, 2008
Apiales	Araliaceae	<i>Eleutherococcus senticosus</i>	AB, IM	R	Won et al. 2008
Apiales	Araliaceae	<i>Panax quinquefolium</i>	GP	W	Abdel-Tawwab, 2012
Apiales	Umbrelliferae	<i>Carum carvi</i>	GP	L	Abdel-Tawwab, 2011
Arecales	Areaceae	<i>Area catechu</i>	AP	L	Caruso et al. 2013
Arecales	Areaceae	<i>Phoenix dactylifera</i>	NA	L	hoseinfar 2015
Asparagales	Amaryllidaceae	<i>Allium sativum</i>	AB, AP, IM, GP	W	Sahu et al. 2007; Aly et al. 2008; Nya and Austin, 2009; Militz et al. 2013a,b
Asparagales	Amaryllidaceae	<i>Allium tuberosum</i>	AB, GP	L	Rattanachaikunsopon and Phumkhachorn, 2009a
Asparagales	Xanthorrhoeaceae	<i>Aloe vera</i>	AB	W	Kim et al. 1999; Alishani et al. 2010
Asterales	Asteraceae	<i>Ageratum conyzoides</i>	NA	L	Caruso et al. 2013
Asterales	Asteraceae	<i>Artemisia annua</i>	AP	L	Martins et al. 2002
Asterales	Asteraceae	<i>Artemisia argyi</i>	AB, AF, AP	NA	Ekanem and Brisibe (2010)
Asterales	Asteraceae	<i>Artemisia capillaries</i>	AB, GP	W	Ji et al. 2007b
Asterales	Asteraceae	<i>Artemisia cina</i>	AB, GP	W	Abdelhadi et al. 2010
Asterales	Asteraceae	<i>Artemisia vulgaris</i>	AB, AV, AP	NA	El-Deen and Mohamed, 2009
Asterales	Asteraceae	<i>Aucklandia lappa</i>	AV	NA	Xue-Gang et al. 2013
Asterales	Asteraceae	<i>Austroeupeatorium inulifolium</i>	NA	NA	Caruso et al. 2013
Asterales	Asteraceae	<i>Chamaemelum nobile</i>	AB	NA	Syahidah et al; 2015
Asterales	Asteraceae	<i>Chrysanthemum cinerariaefolium</i>	AP, IM, GP	NA	Harikrishnan et al. 2010d
Asterales	Asteraceae	<i>Cosmos caudatus</i>	AB	NA	Caruso et al. 2013, Syahidah et al. 2015
Asterales	Asteraceae	<i>Echinacea purpura</i>	AB, AP	L, F	Aly and Mohamed, 2010; Caruana et al. 2012
Asterales	Asteraceae	<i>Eclipta alba</i>	AB, GP	L,S	Christytabpita et al. 2007
Asterales	Asteraceae	<i>Erechtites valerianifolia</i>	NA	NA	Caruso et al. 2013
Asterales	Asteraceae	<i>Eupatorium fortunei</i>	AP	S	Huang et al. 2013
Asterales	Asteraceae	<i>Gynura procumbens</i>	NA	NA	Caruso et al. 2013
Asterales	Asteraceae	<i>Lactuca indica</i>	AB, IM	NA	Harikrishnan et al. 2011c
Asterales	Asteraceae	<i>Matricaria chamomilla</i>	AB, GP	NA	Abdelhadi et al. 2010
Asterales	Asteraceae	<i>Mikania scandens</i>	NA	NA	Caruso et al. 2013
Asterales	Asteraceae	<i>Siegesbeckia glabrescens</i>	AB, IM	W	Harikrishnan et al. 2012b
Asterales	Asteraceae	<i>Spagneticola calendulacea</i>	AB	NA	Direkbysarakom et al. 2004
Asterales	Asteraceae	<i>Tagetes erecta</i>	AB, AV	L	Caruso et al. 2013
Asterales	Asteraceae	<i>Tithonia diversifolia</i>	NA	NA	Caruso et al. 2013
Asterales	Asteraceae	<i>Tridax procumbens</i>	AV, GP	W	Sivasankar et al. 2015
Asterales	Asteraceae	<i>Vernonia amygdaluna</i>	AF	NA	Syahidah et al; 2015
Brassicales	Caricaceae	<i>Carica papaya</i>	AP, GP	NA	Ekanem et al. 2004
Brassicales	Moringaceae	<i>Moringa oleifera</i>	NA	W	Caruso et al. 2013

Caryophyllales	Amaranthaceae	<i>Acyranthes aspera</i>	AB, IM	NA	Vasudeva-Rao et al. 2006
Caryophyllales	Amaranthaceae	<i>Alteranthera sessilis</i>	GP	L, S, Fr	Radhakrishnan et al. 2014
Caryophyllales	Amaranthaceae	<i>Kochia scoparia</i>	AP	NA	Lu et al. 2012
Caryophyllales	Amaranthaceae	<i>Suaeda maritima</i>	AP, IM	NA	Harikrishnan et al. 2011c
Caryophyllales	Cactaceae	<i>Opuntia stricta</i>	NA	L, F	Khem, 2015
Caryophyllales	Caryophyllaceae	<i>Stellaria aquatica</i>	AB, AV	NA	Shangliang et al. 1990 (siva)
Caryophyllales	Molluginaceae	<i>Glinus oppositifolius</i>	AV	Fr	Sivasankar et al. 2015
Caryophyllales	Plumbaginaceae	<i>Aegialitis rotundifolia</i>	AB	L, W	Choudhury et al. 2005
Caryophyllales	Polygonaceae	<i>Polygonum hydropipiper</i>	AB, AV, AF	B	Direkbysarakom et al. 2004
Caryophyllales	Polygonaceae	<i>Rheum officinale</i>	AB, AV, GP	NA	Xie et al. 2008
Caryophyllales	Polygonaceae	<i>Rumex obtusifolius</i>	AP	L	Caruana et al. 2012
Caryophyllales	Portulacaceae	<i>Portulaca oleracea</i>	AB	F	Direkbysarakom et al. 2004
Commelinales	Pontederiaceae	<i>Eichhornia crassipes</i>	AB	NA	Caruso et al. 2013; Chang et al. 2013
Cucurbitales	Cucurbitaceae	<i>Cucurbita pepo</i>	NA	W	Caruso et al. 2013
Cucurbitales	Cucurbitaceae	<i>Mormodica charantia</i>	AV	R	Balasubramanian et al. 2007
Cucurbitales	Cucurbitaceae	<i>Mormodica cochinchinensis</i>	AP	L	Wu et al. 2011
Cyatheales	Cyatheaceae	<i>Cyathea kanehirae</i>	AB, IM	L	Yeh and Chen, 2009
Dipsacales	Caprifoliaceae	<i>Lonicera japonica</i>	AB, AV, IM	L	Direkbysarakom et al. 2006; Ardò et al. 2008
Dryopteridales	Dryopteridaceae	<i>Dryopteris crassirizhoma</i>	AP	NA	Lu et al. 2012
Ericales	Primulaceae	<i>Aegiceras corniculatum</i>	AB	NA	Choudhury et al. 2005
Ericales	Theaceae	<i>Camellia sinensis</i>	AB, AP, GP	NA	Sheikhzadeh et al. 2011
Ericales	Lecythidaceae	<i>Cariniana legalis</i>	AB	NA	Castro et al. 2008
Ericales	Balsaminaceae	<i>Impatiens biflora</i>	AB, AV	NA	Direkbysarakom et al. 2004
Ericales	Primulaceae	<i>Lysimachia christinae</i>	AP	NA	Huang et al. 2013
Ericales	Styracaceae	<i>Styrax japonica</i>	AB	S	Harikrishnan et al. 2011g
Fabales	Fabaceae	<i>Albizia saman</i>	NA	NA	Caruso et al. 2013
Fabales	Fabaceae	<i>Astragalus membranaceus</i>	AB, IM	L	Pan et al. 2013
Fabales	Fabaceae	<i>Caesalpinia sappan</i>	AP	NA	Huang et al. 2013
Fabales	Fabaceae	<i>Cassia alata</i>	AV	NA	Sivasankar et al. 2015
Fabales	Fabaceae	<i>Cassia fistula</i>	AV	NA	Sivasankar et al. 2015
Fabales	Fabaceae	<i>Cynometra iripa</i>	AB	NA	Choudhury et al. 2005
Fabales	Fabaceae	<i>Falcataria moluccana</i>	NA	NA	Caruso et al. 2013
Fabales	Fabaceae	<i>Gliricidia sepium</i>	NA	NA	Caruso et al. 2014
Fabales	Fabaceae	<i>Glycine max</i>	NA	R	Caruso et al. 2015
Fabales	Fabaceae	<i>Koompassia malaccensis</i>	NA	NA	Caruso et al. 2016
Fabales	Fabaceae	<i>Leucaena leucocephala</i>	NA	L, R	Caruso et al. 2017
Fabales	Fabaceae	<i>Leucaena glauca</i>	AP	W	Direkbysarakom et al. 2004; Caruso et al. 2013
Fabales	Fabaceae	<i>Lupinus perennis</i>	NA	NA	Awad and Austin, 2010
Fabales	Fabaceae	<i>Mimosa pudica</i>	AV	NA	Sivasankar et al. 2015
Fabales	Fabaceae	<i>Mucuna pruriens</i>	AP	W	Ekanem et al. 2004
Fabales	Fabaceae	<i>Parkia speciosa</i>	NA	L	Caruso et al. 2013
Fabales	Fabaceae	<i>Psoralea corylifolia</i>	AP, GP	L	Ling et al. 2003
Fabales	Fabaceae	<i>Senna alata</i>	AB, AV	NA	Caruso et al. 2013
Fabales	Fabaceae	<i>Senna siamea</i>	NA	NA	Caruso et al. 2013
Fabales	Fabaceae	<i>Sesbania grandiflora</i>	NA	NA	Caruso et al. 2013
Fabales	Fabaceae	<i>Sophora alopecuroides</i>	AB, AP	NA	Yi et al. 2012
Fabales	Fabaceae	<i>Sophora flavescens</i>	AB, AP	NA	Caruana et al. 2012; Wu et al. 2013
Fabales	Fabaceae	<i>Tamarindus indica</i>	NA	L, R	Caruso et al. 2013
Fabales	Fabaceae	<i>Tephrosia purpurea</i>	NA	NA	Sivasankar et al. 2015
Fabales	Fabaceae	<i>Trifolium pratense</i>	GP	S	Syahidah et al. 2015
Fabales	Polygalaceae	<i>Polygala tenuifolia</i>	AP	NA	Lu et al. 2012
Fabales	Quillajaceae	<i>Quillaja saponaria</i>	GP	NA	Francis et al. 2005
Fagales	Betulaceae	<i>Alnus firma</i>	AB	NA	Harikrishnan et al. 2011i
Gentianales	Apocynaceae	<i>Anathoda vasica</i>	AB, IM	L	Minomol 2005
Gentianales	Apocynaceae	<i>Calotropis gigantea</i>	AB, IM	L	Olusola et al. 2013
Gentianales	Apocynaceae	<i>Catharanthus roseus</i>	AV	L	Sivasankar et al. 2015
Gentianales	Apocynaceae	<i>Daemia extensa</i>	AB	L	Jinish, 2002
Gentianales	Apocynaceae	<i>Plumeria rubra</i>	NA	NA	Caruso et al. 2013
Gentianales	Rubiaceae	<i>Coffea arabica</i>	NA	NA	Abdel, 2015
Gentianales	Rubiaceae	<i>Morinda citrifolia</i>	NA	NA	Caruso et al. 2013
Lamiales	Acanthaceae	<i>Andrographis paniculata</i>	AB, GP	NA	Rattanachaikunsopon and Phumkhachorn, 2009; Caruso et al. 2009
Lamiales	Acanthaceae	<i>Clinacanthus nutans</i>	AV	W	Direkbysarakom et al. 1998
Lamiales	Acanthaceae	<i>Hygrophila spinosa</i>	AB, IM	L	Raja et al. 2012
Lamiales	Lamiaceae	<i>Leucas lavandulifolia</i>	NA	NA	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Mentha piperita</i>	NA	W	Abasali and Mohamad, 2010
Lamiales	Lamiaceae	<i>Ocimum americanum</i>	NA	NA	Sivasankar et al. 2015
Lamiales	Lamiaceae	<i>Ocimum basilicum</i>	GP	W	Syahidah et al. 2015
Lamiales	Lamiaceae	<i>Ocimum sanctum</i>	AB, IM, GP	NA	Logambal et al. 2000; Pajaraj et al. 2011

Lamiales	Lamiaceae	<i>Ocimum tenuiflorum</i>	NA	NA	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Origanum monites</i>	AF	W	Syahidah et al. 2015
Lamiales	Lamiaceae	<i>Origanum heracleoticum</i>	AB	W	Zheng et al. 2007
Lamiales	Lamiaceae	<i>Origanum minutiflorum</i>	AP	Fr	Karagouni et al. 2005
Lamiales	Lamiaceae	<i>Origanum onites</i>	AF	S	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Origanum vulgare</i>	AB, GP	NA	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Prunella vulgaris</i>	AP, IM	R	Harikrishnan et al. 2011d
Lamiales	Lamiaceae	<i>Rosmarinus officinalis</i>	AB, AP	NA	Abutbul et al. 2004
Lamiales	Lamiaceae	<i>Satureja khuzistanica</i>	IM	NA	Khansari et al. 2013
Lamiales	Lamiaceae	<i>Satureja bachtarica</i>	AB	B	Pirbalouti et al. 2011
Lamiales	Lamiaceae	<i>Satureja thymbra</i>	AF	B	Syahidah et al. 2015
Lamiales	Lamiaceae	<i>Scutellaria baicalensis</i>	AB, IM	B	Harikrishnan et al. 2012b; Pan et al. 2013
Lamiales	Lamiaceae	<i>Plectranthus scutellarioides</i>	NA	W	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Tectona grandis</i>	NA	NA	Caruso et al. 2013
Lamiales	Lamiaceae	<i>Thymbra spicata</i>	AF	L, B	Syahidah et al. 2015
Lamiales	Lamiaceae	<i>Zataria multiflora</i>	AF	NA	Soltani et al. 2010
Lamiales	Oleaceae	<i>Fructus forsythiae</i>	AB	NA	Pan et al. 2013
Lamiales	Oleaceae	<i>Nyctanthes arbortristis</i>	AB, IM	NA	Kibubakaran et al. 2010
Lamiales	Oleaceae	<i>Olea europea</i>	AV	NA	Micol et al. 2005
Lamiales	Plantaginaceae	<i>Picrorhiza kurrooa</i>	AB, AV, IM	L	Raja et al. 2012
Lamiales	Pedaliaceae	<i>Sesamum indicum</i>	NA	L	Dada et al. 2014
Lamiales	Verbenaceae	<i>Lantana camara</i>	AV	L, S, B	Balasubramanian et al. 2007
Laurales	Lauraceae	<i>Cinnamomum cassia</i>	AB, AP	W	Ji et al. 2012
Laurales	Lauraceae	<i>Cinnamomum verum</i>	AB, GP	NA	Talpur and Ikhwanuddin et al. 2013
Laurales	Lauraceae	<i>Cinnamomum zeylanicum</i>	AB	L	Ahmad et al. 2011
Laurales	Lauraceae	<i>Laurus nobilis</i>	AB, IM	L, B	Bilen and Bulut, 2010
Laurales	Lauraceae	<i>Lindera aggregata</i>	AP	NA	Ji et al. 2012
Laurales	Siparunaceae	<i>Siparuna guianensis</i>	AB	NA	Castro et al. 2008
Liliales	Melanthiaceae	<i>Paris polyphylla</i>	NA	NA	Wang et al. 2010a
Magnoliales	Annonaceae	<i>Annona muricata</i>	AB	L	Caruso et al. 2013
Magnoliales	Annonaceae	<i>Xylopiya aethiopica</i>	NA	NA	Okeke et al. 2001
Magnoliales	Magnoliaceae	<i>Magnolia officinalis</i>	AV, AF	NA	Caruana et al. 2012; Huang et al. 2015
Magnoliales	Myristicaceae	<i>Myristica fragans</i>	AB, IM, GP	L	Sivaram et al. 2004
Magnoliales	Myristicaceae	<i>Virola sebifera</i>	AB	NA	Castro et al. 2008
Malpighiales	Calophyllaceae	<i>Calophyllum inophyllum</i>	AV	NA	Sivasankar et al. 2015
Malpighiales	Euphorbiaceae	<i>Acalypha indica</i>	AB, AV, IM	NA	Raja et al. 2012
Malpighiales	Euphorbiaceae	<i>Acalypha australis</i>	AV	L	Direkbyarakom et al. 2004
Malpighiales	Euphorbiaceae	<i>Actinostemon concolor</i>	AB	W	Castro et al. 2008
Malpighiales	Euphorbiaceae	<i>Croton floribundus</i>	AB	NA	Castro et al. 2008
Malpighiales	Euphorbiaceae	<i>Croton zambesicus</i>	AV	NA	Sivasankar et al. 2015
Malpighiales	Euphorbiaceae	<i>Euphorbia antiquorum</i>	NA	NA	Caruso et al. 2013
Malpighiales	Euphorbiaceae	<i>Euphorbia fishceriana</i>	AF, AP	W	Zhang et al. 2014
Malpighiales	Euphorbiaceae	<i>Euphorbia hirta</i>	AB	NA	Pratheepa and Sukumaran, 2011; Huang et al. 2015
Malpighiales	Euphorbiaceae	<i>Euphorbia plumerioides</i>	NA	NA	Caruso et al. 2013
Malpighiales	Euphorbiaceae	<i>Euphorbia thymifolia</i>	AB	NA	Direkbyarakom et al. 2004
Malpighiales	Euphorbiaceae	<i>Exoecaria agallocha</i>	AB	W	Dhayanithi et al. 2012
Malpighiales	Euphorbiaceae	<i>Hura crepitans</i>	AV	L	Sivasankar et al. 2015
Malpighiales	Euphorbiaceae	<i>Manihot esculenta</i>	NA	NA	Caruso et al. 2013
Malpighiales	Euphorbiaceae	<i>Sapium sebiferum</i>	AV	NA	Direkbyarakom et al. 2004
Malpighiales	Euphorbiaceae	<i>Tetracarpidium conophorum</i>	AB, IM, GP	Fr	Olusola et al. 2013
Malpighiales	Hypericaceae	<i>Cratoxylum formosum</i>	AB, IM, GP	NA	Rattanachaiakunsopon and Phumkhachorn, 2010c
Malpighiales	Phyllanthaceae	<i>Antidesma bunius</i>	NA	W	Caruso et al. 2013
Malpighiales	Phyllanthaceae	<i>Phyllanthus acidus</i>	NA	L, F	Caruso et al. 2013
Malpighiales	Phyllanthaceae	<i>Phyllanthus amarus</i>	AB, AV	L	Balasubramanian et al. 2007
Malpighiales	Phyllanthaceae	<i>Phyllanthus niruri</i>	GP	L	Citarasu et al. 2002
Malpighiales	Phyllanthaceae	<i>Phyllanthus debelis</i>	AB, AV	L, B, Fr	Sivasankar et al. 2015
Malpighiales	Phyllanthaceae	<i>Phyllanthus emblica</i>	AV, IM	NA	Sivasankar et al. 2015
Malpighiales	Phyllanthaceae	<i>Phyllanthus reticulatus</i>	AB, AV	W	Sivasankar et al. 2015
Malpighiales	Phyllanthaceae	<i>Phyllanthus urinaria</i>	AB, AV	R, F, S	Caruso et al. 2013
Malpighiales	Phyllanthaceae	<i>Sauropus androgynus</i>	GP	NA	Putra et al. 2013
Malvales	Malvaceae	<i>Hibiscus rosa-sinensis</i>	NA	NA	Caruso et al. 2013
microalgae		<i>Euglena viridis</i>	AB, IM	NA	Das, 2009
Mucorales	Mucoraceae	<i>Massa medicata</i>	AB, GP	NA	Ji et al. 2007a,b
Myrtales	Combretaceae	<i>Terminalia bellerica</i>	AB	NA	Jinsh 2002
Myrtales	Combretaceae	<i>Terminalia cattapa</i>	AB, AP	S	Chitmanat et al. 2005; Purivirojkul, 2012
Myrtales	Lythraceae	<i>Punica granatum</i>	AB, AV, AP, IM,	NA	Harikrishnan et al. 2010e

			GP		
Myrtales	Myrtaceae	<i>Calyptranthes clusiifolia</i>	AB	W	Castro et al. 2008
Myrtales	Myrtaceae	<i>Myrcia tomentosa</i>	AB	NA	Castro et al. 2008
Myrtales	Myrtaceae	<i>Psidium guajava</i>	AB, AV	NA	Pachanawan et al. 2008
Myrtales	Myrtaceae	<i>Syzygium aromaticum</i>	NA	NA	Rattanachaikunsopon and Phumkachorn, 2010; Abd El-Galil, 2012
Myrtales	Onagraceae	<i>Epilobium hirsutum</i>	AB	NA	Pakravan et al. 2011
Myrtales	Onagraceae	<i>Oenothera biennis</i>	AB, AV	L, R	Shangliang et al. 1990 (sriva)
Pinales	Pinaceae	<i>Pinus massoniana</i>	AB, AF, AP	L, R	Direkbysarakom et al. 2004
Pinales	Pinaceae	<i>Pseudolarix kaempferi</i>	AP	NA	Ji et al. 2012
Piperales	Aristolochiaceae	<i>Aristolochia indica</i>	AV	NA	Sivasankar et al. 2015
Piperales	Piperaceae	<i>Piper betle</i>	AB, AF	NA	Caruso et al. 2013
Piperales	Piperaceae	<i>Piper longum</i>	GP	NA	Citarasu et al. 2002
Piperales	Piperaceae	<i>Piper guineense</i>	AP	S	Ekanem et al. 2004
Piperales	Piperaceae	<i>Piper nigrum</i>	IM	R	Caruso et al. 2013
Poales	Poaceae	<i>Cynodon dactylon</i>	AB, AV, AP, IM, GP	NA	Balasubramanian et al. 2007, 2008a,b; Kaleeswaran et al. 2011
Poales	Poaceae	<i>Oryza sativa</i>	NA	NA	Caruso et al. 2013
Poales	Poaceae	<i>Panicum repens</i>	NA	R	Caruso et al. 2014
Poales	Poaceae	<i>Setaria barbata</i>	NA	NA	Caruso et al. 2015
Proteales	Nelumbonaceae	<i>Nelumbo nucifera</i>	NA	NA	Liu et al. 2004; Shao et al. 2004
Ranunculales	Menispermaceae	<i>Tinospora cordifolia</i>	AB, IM	Fr	Sudhakaran et al. 2006; Alexander et al. 2010
Ranunculales	Menispermaceae	<i>Tinospora crispa</i>	NA	W	Sivasankar et al. 2015
Ranunculales	Menispermaceae	<i>Tinospora tuberculata</i>	NA	NA	Caruso et al. 2013
Ranunculales	Papaveraceae	<i>Chelidonium majus</i>	AP	L	Yao et al. 2011
Ranunculales	Papaveraceae	<i>Macleaya microcarpa</i>	AP	NA	Wang et al. 2010b
Ranunculales	Ranunculaceae	<i>Cimifuga fetida</i>	AP	NA	Wu et al. 2011
Ranunculales	Ranunculoideae	<i>Nigella sativa</i>	NA	L, B, Fr	Dorucu et al. 2009
Ranunculales	Ranunculaceae	<i>Rhizoma cimigufae</i>	AP	NA	Wu et al. 2011
Rosales	Moraceae	<i>Artocarpus altilis</i>	NA	W	Caruso et al. 2013
Rosales	Moraceae	<i>Artocarpus heterophyllus</i>	NA	NA	Caruso et al. 2014
Rosales	Moraceae	<i>Ficus benghalensis</i>	IM	Fr	Olusola et al. 2013
Rosales	Moraceae	<i>Ficus septica</i>	AB	NA	Caruso et al. 2013
Rosales	Moraceae	<i>Morus alba</i>	AV, AP	L	Sivasankar et al. 2015
Rosales	Rosaceae	<i>Crataegi fructus</i>	AB, GP	NA	Ji et al. 2007b; Takaoka et al. 2011
Rosales	Rosaceae	<i>Eriobotrya japonica</i>	AB	NA	Kim et al. 2011
Rosales	Rosaceae	<i>Prunus amygdalus</i>	AP	L	Wu et al. 2011
Rosales	Urticaceae	<i>Urtica dioica</i>	AB, IM	L	Awad and Austin, 2010
Salviniales	Salviniaceae	<i>Salvinia adnata</i>	NA	NA	Caruso et al. 2013
Santalales	Santalaceae	<i>Santalum album</i>	AP	NA	Tu et al. 2013
Santalales	Santalaceae	<i>Viscum album</i>	AB, IM	NA	Sharma et al. 2010; Park and Choi, 2012
Sapindales	Anacardiaceae	<i>Anacardium occidentale</i>	NA	NA	Caruso et al. 2013
Sapindales	Anacardiaceae	<i>Cotinus coggyria</i>	GP	NA	Bilen et al. 2011
Sapindales	Anacardiaceae	<i>Galla chinensis</i>	AV	NA	Direkbysarakom et al. 2004
Sapindales	Anacardiaceae	<i>Mangifera indica</i>	AB, IM	NA	Sahu et al. 2007b; Awad and Austin, 2010; Awad et al. 2011
Sapindales	Burseraceae	<i>Commiphora myrrha</i>	AP	L	Syahidah et al. 2015
Sapindales	Meliaceae	<i>Azadirachta indica</i>	AB	NA	Talpur and Ikhwanuddin, 2013
Sapindales	Meliaceae	<i>Dysoxylum gaudichaudianum</i>	NA	NA	Caruso et al. 2013
Sapindales	Meliaceae	<i>Melia azedarach</i>	AV, AP	NA	Caruso et al. 2013; Sivasankar et al. 2015
Sapindales	Meliaceae	<i>Toona sinensis</i>	AB, IM	L	Wu et al. 2010
Sapindales	Rutaceae	<i>Aegle marmelos</i>	AB, AV, AP	NA	Balasubramanian et al. 2007; Pratheepa et al. 2010
Sapindales	Rutaceae	<i>Murraya paniculata</i>	NA	W	Caruso et al. 2013
Sapindales	Rutaceae	<i>Ruta graveolens</i>	AF	R	Hashemi-Karouei et al. 2011; Caruso et al. 2013
Sapindales	Rutaceae	<i>Zanthoxylum schinifolium</i>	AP, IM, GP	L	Harikrishnan et al. 2010d
Sapindales	Simaroubaceae	<i>Brucea javanica</i>	NA	W	Wang et al. 2011
Solanales	Convolvulaceae	<i>Cuscuta chinensis</i>	AP	R	Huang et al. 2013
Solanales	Convolvulaceae	<i>Ipomoea aquatica</i>	NA	R	Caruso et al. 2013
Solanales	Convolvulaceae	<i>Ipomoea batatas</i>	NA	R	Caruso et al. 2013
Solanales	Convolvulaceae	<i>Merremia tomentosa</i>	AB	R	Castro et al. 2008
Solanales	Solanaceae	<i>Brugmansia suaveolens</i>	AB	NA	Caruso et al. 2013
Solanales	Solanaceae	<i>Capsicum frutescens</i>	AP	NA	Ling et al. 2012
Solanales	Solanaceae	<i>Physalis angulata</i>	NA	NA	Caruso et al. 2013
Solanales	Solanaceae	<i>Solanum nigurum</i>	NA	NA	Rajendiran et al. 2008
Solanales	Solanaceae	<i>Solanum suratense</i>	AB	R	Jinish, 2002
Solanales	Solanaceae	<i>Solanum trilobatum</i>	AB, IM, GP	NA	Divyagnaneswari et al. 2007
Solanales	Solanaceae	<i>Withania somnifera</i>	AB, AV, IM, GP	NA	Sharma et al. 2010
Vitales	Vitaceae	<i>Cayrathia japonicus</i>	AV	NA	Direkbysarakom et al. 2004

Vitales	Vitaceae	<i>Cissus quadrangularis</i>	GP	NA	Radhakrishnan et al. 2014
Zingiberales	Zingiberaceae	<i>Curcuma longa</i>	AB, AF, IM	NA	Sahu et al. 2008; Caruso et al. 2013
Zingiberales	Zingiberaceae	<i>Curcuma mangga</i>	AB	NA	Caruso et al. 2013
Zingiberales	Zingiberaceae	<i>Curcuma zanthorrhiza</i>	NA	NA	Caruso et al. 2014
Zingiberales	Zingiberaceae	<i>Curcuma zeodaria</i>	AB, AF	NA	Caruso et al. 2015
Zingiberales	Zingiberaceae	<i>Etilingera hemisphaerica</i>	NA	NA	Caruso et al. 2016
Zingiberales	Musaceae	<i>Musa acuminata</i>	NA	NA	Caruso et al. 2017
Zingiberales	Musaceae	<i>Musa balbisiana</i>	NA	NA	Caruso et al. 2018
Zingiberales	Musaceae	<i>Musa paradisiaca</i>	NA	NA	Caruso et al. 2019
Zingiberales	Zingiberaceae	<i>Zingiber officinale</i>	AB, AV, AF, AP, IM, GP	NA	Düğenci et al. 2003; Nya and Austin, 2009a; Haghghi and Rohani, 2013
Algae	red	<i>Asparagopsis taxiformis</i>	AB, AF, AP	NA	Genovese et al. 2012; Hutson et al; 2012; Manilal et al. 2012
Algae	brown	<i>Himantalia elongata</i>	AB	NA	Cox et al. 2010
Algae	brown	<i>Laminaria digitata</i>	AB	NA	Dubber and Harder, 2008
Algae	red	<i>Ceramium rubrum</i>	AB	NA	Dubber and Harder, 2009
Algae	red	<i>Gracilaria edulis</i>	AB	NA	Kolanjinathan et al. 2009
Algae	red	<i>Gracilaria fisheri</i>	NA	NA	harik 2011
Algae	red	<i>Gracilaria tenuistipitata</i>	AB, AV	NA	Sirirustananun et al. 2011
Algae	red	<i>Gelidium amansii</i>	AB, IM	NA	Fu et al. 2007
Algae	brown	<i>Sargassum fusiforme</i>	AB, IM	NA	Huang et al. 2006
Algae	brown	<i>Sargassum duplicatum</i>	AB, IM	NA	Yeh et al. 2006
Algae	red	<i>Porphyridium cruentum</i>	AB	NA	Diaz-Rosales et al. 2008
Algae	green	<i>Ulva sp.</i>	AP	NA	Hutson et al. 2012
Mushroom		<i>Citrus paradisi</i>	AB	NA	Oladosu-Ayayi et al. 2013
Mushroom		<i>Ganoderma lucidum</i>	AB, IM	NA	Olusola et al. 2013
Mushroom		<i>Hericium erinaceum</i>	AP, IM	NA	Harikrishnan et al. 2011b
Mushroom		<i>Inonotus obliquus</i>	AB	NA	Harikrishnan et al. 2012a
Mushroom		<i>Volvariella volvacea</i>		NA	Caruso et al. 2013

Table 1. Plants, algae and mushrooms used or studied for potential application in aquaculture. AV : antiviral, NA : not available, AB : antibacterial, IM : immunostimulant, AF : antifungal, AP : antiparasitic, GP : growth promoter. R : root, L : leaves, W : whole plant, Fr : fruit, S : seeds, F : flowers.

3.1. Plant orders most frequently used in aquaculture

Plants from the order Lamiales (family Lamiaceae) were the most often studied plants (12%), followed by the Fabales (family Fabaceae, 11%), Asterales (family Asteraceae, 10%) and Malpighiales (family Euphorbiaceae et Phyllanthaceae, 10%) (Figure 2).

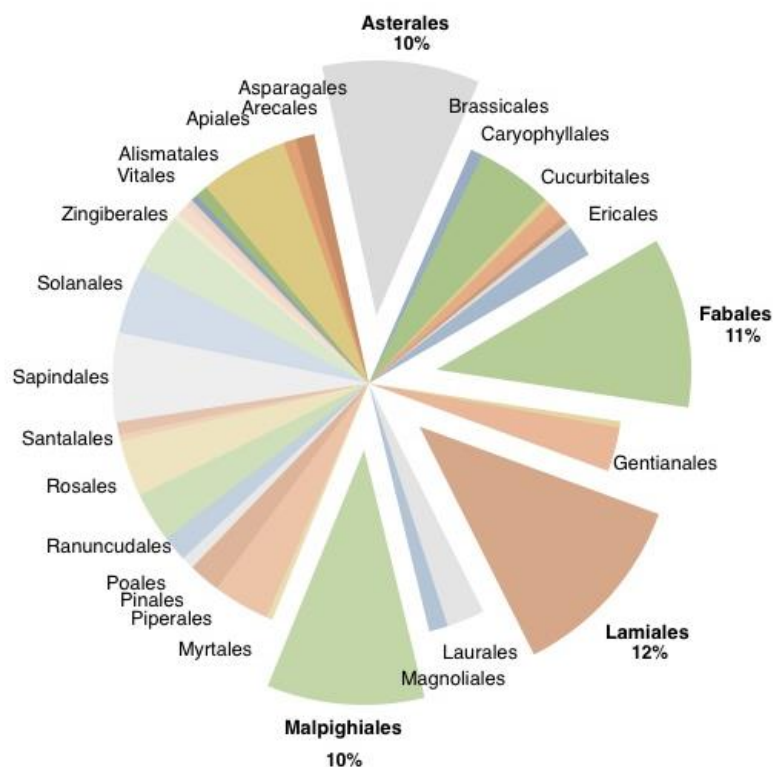


Figure 2. Plant orders used in aquaculture.

Lamiales

The Lamiales order comprises of about 20 families of dicotyledonous flowering plants (Allaby, 2006). The Lamiaceae plants are frequently aromatic and include many culinary herbs such as basil (*Ocimum sp.*), mint (*Mentha sp.*), oregano (*Origanum sp.*) rosemary (*Rosmarinus officinalis*) and savory (*Satureja sp.*). Lamiaceae plants are widely used in traditional medicine for treatment of different diseases such as colds, headaches, stomach disorders, inflammation, and heart disease (Pattayanak et al. 2010). Several essential reported to display antimicrobial, antispasmodic, carminative and antiviral activities (Mimika-Duzic and Boizin, 2008). Lamiaceae plants contain a wide range of bioactive metabolites which include numerous monoterpenes and sesquiterpenes such as linalool, geraniol, eugenol, ocimene, carvacrol, p-cymene, thymol and myrcene and flavonoids like luteolin first isolated from *Salvia tomentosa* (Mimika-Duzic and Boizin, 2008; Bower et al. 2014; Malmir et al. 2015) (Figure 3). Plants from the family Lamiaceae also contain several bioactive fatty acids such as palmitic acid and myristic acid and phenolic actives like rosmarinic acid (Pattayanak et al. 2010). Rattanachaikunsopon and Phumkhachorn (2010) and Abd-El-Galil (2012) showed that antibacterial activity of carvacrol against *Edwardsiella tarda* and *Tenacibaculum maritimum* was enhanced when administered with cymene (Figure 3). Gormez and Diler (2014) showed that essential oils of *Origanum onites* and *Thymbra spicata* were more bioactive against the fungus *Saprolegnia parasitica* than thymol or carvacrol alone. The latter studies indicate that synergistic effects between different bioactive compounds may exist and enhance the plant bioactivities. Hao et al. (2012) found that palmitic acid was effective against the monogenean parasite *D. intermedium* in goldfish (*Carassius auratus*).

Fabales

The Fabales include the family Fabaceae, commonly known as pea or bean family. The Fabaceae is the third-largest plant family as it includes more than 12.000 plants (Allaby, 2006). Several plants from the family Fabaceae are used in traditional medicine for treatments against diarrhea, parasite infections, inflammations, rheumatism and ulcers (Molares and Ladio, 2011). Several plants from the Fabaceae family (*Albizia saman*, *Falcataria moluccana*, *Gliricidia sepium*, *Koompassia malaccensis*, *Leucaena leucocephala*, *L. glauca*, *Parkia speciosa*, *Senna alata*, *S. siamea*, *Sesbania grandiflora* and *Tamarindus indica*) are used by Indonesian fish farmers to improve fish fitness and treat fish infections (Caruso et al. 2013). The plants from the genus *Sophora* contain many phytoconstituents with pharmacological and therapeutic properties including, antioxidant, anticancer, antimicrobial, antiviral and anti-inflammatory (Abdelhady et al. 2015). Several polyphenolic compounds have been identified in *Sophora japonica* like tamarixetin, sissotrin, rutin, gallic acid and quercetin (Abdelhady et al. 2015) (Figure 3). Liu et al. (2011) characterised the alkaloids matrine, oxymatrine, sophoridine, oxysophocarpine, and sophocarpine from *Sophora flavescens*. Wu et al. (2013) showed that a diet supplemented with *Sophora flavescens* enhanced the non-specific immune system of Nile tilapia (*Oreochromis niloticus*) and increased disease resistance against *Streptococcus agalactiae*.

Asterales

Asterales is an order of dicotyledonous flowering plants that includes the large family Asteraceae, commonly known as the daisy family. The Asteraceae family contains more than 23.000 species and most members are herbaceous, but a significant number are also shrubs, vines, or trees (Allaby, 2006).

Many artemisia species are present in the Chinese pharmacopeia, and they have been used as scholeretic, anti-inflammatory and diuretic agents in the treatment of diseases like epidemic hepatitis and fevers (Tang et al. 1992). The use of *Artemisia annua* in Chinese traditional medicine was recorded before 168 BC, and in 1971 artemisinin, a sesquiterpene lactone with antimalarial properties was isolated (Klayman, 1985; Cavar et al. 2012). Artemisinin and its semi-synthetic derivatives are currently used worldwide for the treatment against the malaria parasite *Plasmodium falciparum* (WHO, 2006) (Figure 3). Artemisia plants also contain abundant monoterpenes (eucalyptol, germacrenes, camphor), coumarins (scopoletin) chlorogenic acids (quinic acid, caffeic acid) and flavonoids (luteolin, isovitexin) (Juteau et al. 2002; Efferth et al. 2011; Carbonara et al. 2012, Cavar et al. 2012) (Figure 3). *Artemisia sp.* antibacterial and antiparasitic effects have also been studied against aquaculture pathogens. Ekanem and Bribe (2010) showed the efficacy of ethanol extract of *Artemisia annua* against the monogenean parasite *Heterobranhus longifilis*. Huang et al. (2013) also showed the anthelmintic activity of *Artemisia argyi* against *D. intermedius* in goldfish. El-Deen and Mohamed (2009) showed the efficacy of *Artemisia vulgaris* crude extracts against the ectoparasite *Trichodina sp.* and the bacteria *Aeromonas hydrophila* in Nile tilapia.

Aucklandia lappa is another plant from the Asteraceae family with numerous bioactivities and widely used in traditional medicines for the treatment of asthma, rheumatism, coughs, tuberculosis, and many other diseases. (Zhang et al. 2014; Seo and Shin, 2015). *A. lappa* contains the sesquiterpenes lactones costunolide, dehydrocostus lactone, and alantolactone which are known to display multiple bioactivities (Li et al. 2005; Seo and Shin, 2015). Xue-Gang et al. (2013) showed that *A. lappa* displayed antifungal activity against *Saprolegnia sp.* and *Achlya klebsiana*. Other Asteraceae plants are shown to display interesting bioactivities for their application in aquaculture. Oral administration of *Eclipta alba* in tilapia *Oreochromis mossambicus* enhanced non-specific immune parameters and reduced fish mortality when challenged with *A. hydrophila* (Christyapita et al. 2007). *Siegesbeckia glabrescens* enriched diet also displayed an immunomodulatory effect and increase disease resistance in kelp grouper (*Epinephelus bruneus*) (Harikrishnan et al. 2012b). *Artemisia cina*, *Matricaria chamomilla* and *Tridax procumbens* promoted weight gain and enhanced non-specific immune responses in fish (Abdelhadi et al. 2010; Sivasankar et al. 2015). *Echinacea purpurea* appeared to have immunostimulant, antibacterial and antifungal properties in fish (Aly and Mohamed, 2010; Caruana et al. 2012).

Malpighiales

Malpighiales is a very diverse order, containing the families of flowering plants Euphorbiaceae and Phyllanthaceae. Euphorbiaceae, the spurge family, is constituted mainly of herbs but also includes some shrubs and trees. The spurge family contains numerous species of medicinal plants such as different species from the genus *Euphorbia* or the irritant mangrove *Excoecaria agallocha* (Ernst et al. 2015). The study of the natural products of the *Euphorbia* species started in 1968 with the isolation of a tumour-promoting phorbol-12,13-diester from *Croton tiglium* L., and the description of the anti-tumour activity of a diterpene isolated from *Euphorbia esula* L. in 1976 (Hecker, 1968 ; Kupchan, 1976). More recently, a diterpene drug (ingenol mebutate, Picato®) isolated from *Euphorbia peplus* was released for the treatment of actinic keratosis (Berman, 2012). *Euphorbia sp.* contains several terpenes, such as the ingol-type diterpens euphorantins, flavonoids like quercitrin and tannins (Qi et al. 2014, Trinh and Le, 2014) (Figure 3). Different species of *Euphorbia* have displayed antibacterial, antiviral, antifungal and antiparasitic activities against fish and shellfish pathogens (Direkbusarakom, 2004; Zhang et al. 2014; Huang et al. 2015). The Phyllanthaceae family is a small (about 2000 species) but diverse family, which includes the genus *Phyllanthus*. *Phyllanthus* plants are well known by their potent medicinal activities and are used worldwide (Kumar et al. 2015). Numerous bioactive phytochemicals have been isolated from *Phyllanthus* plants including phyllanthin, phylltetralin, phyllangin, corilangin, gallic acid, methylgallate, and rhamnocitrin, protocatechuic acid, kaempferol 3-O-rutinoside, quercitrin, and rutin (Wei et al. 2004 ; Fang et al. 2008 ; Kumar et al. 2015). *Phyllanthus* have proved to possess a high antibacterial and antiviral potential against pathogens in aquaculture (Balasubramanian et al. 2007; Punitha et al. 2008; Sivasankar et al. 2015).

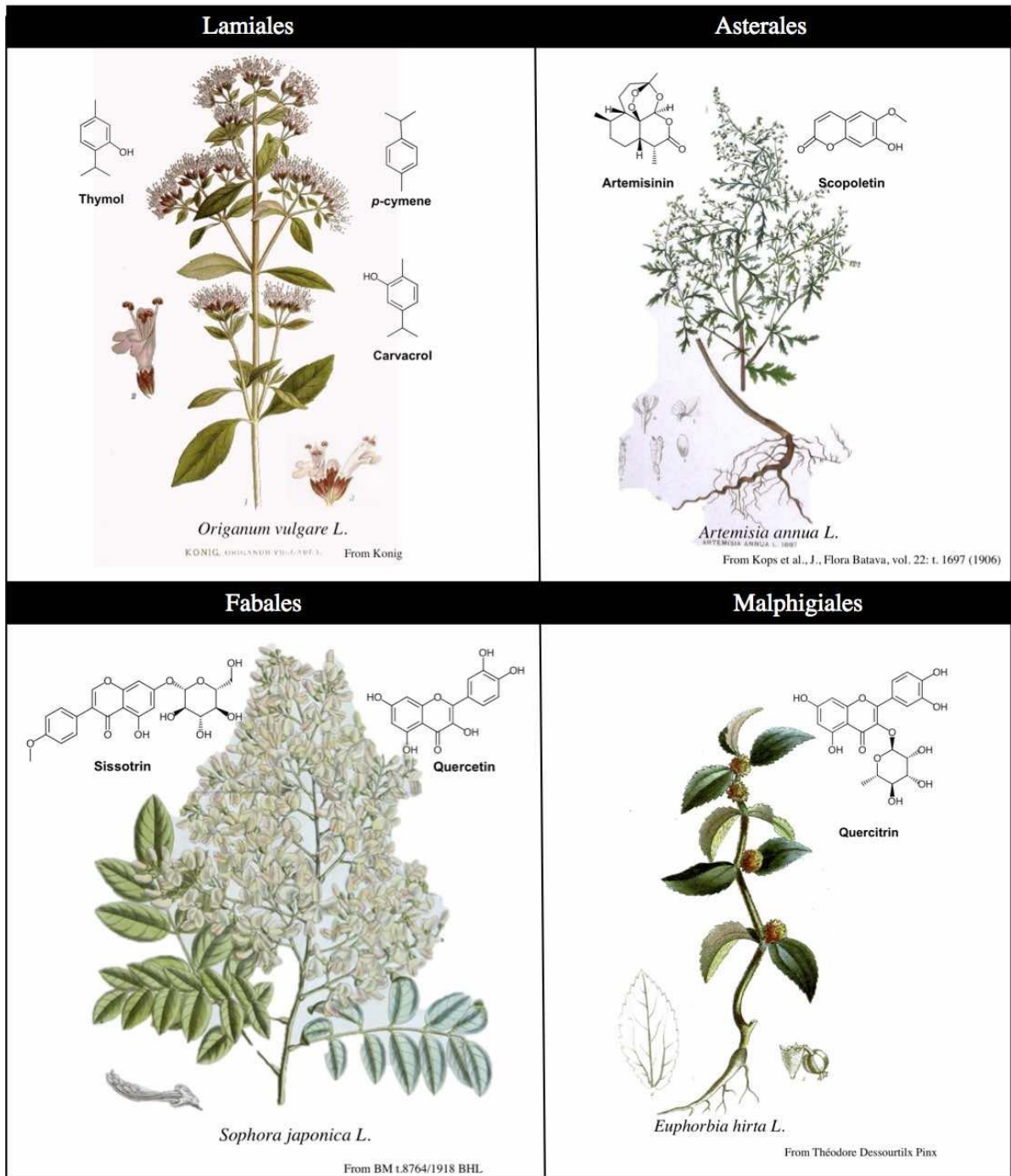


Figure 3. Plant examples and isolated molecules from the most used plant orders in aquaculture.

3.2. Plant species most widely used in aquaculture

The plant species that displayed the highest potential to be used in aquaculture are garlic (*Allium sativum*), pomegranate (*Punica granatum*), bermuda grass (*Cynodon dactylon*), indian ginseng (*Whitania somnifera*) and ginger (*Zingiber officinale*).

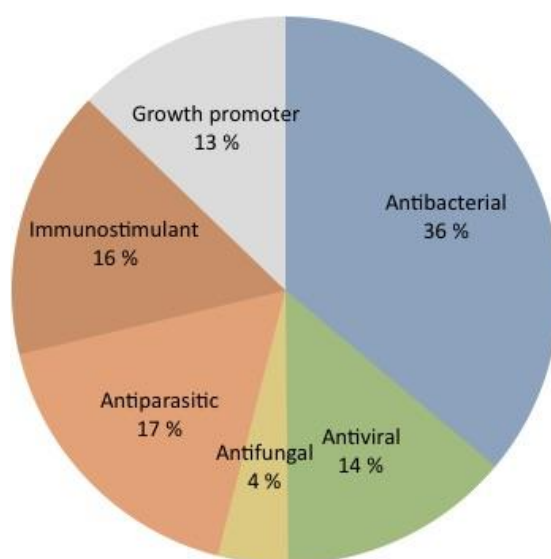
Garlic (Asparagales, Amaryllidaceae) has been used by humans over 7000 years for both culinary and medicinal purposes. Antibacterial, antiparasitic, antioxidant, immunostimulant and growth promoting activities have been observed on fish and shellfish (Lee and Gao, 2012). Some studies have studied the effect of pure garlic components allicin and ajoene in aquaculture and have showed their immunostimulant capacity and their effectiveness against pathogenic fish protozoa *Spironucleus vortens*, *Ichthyophthirius multifiliis* and the bacteria *A. hydrophila* (Nya et al. 2010; Millet et al. 2011; Tanekhy and Fall, 2015). Pomegranate (Myrtales, Lythraceae) has also been used for medical purposes since ancient times. Pomegranates contain numerous phytochemicals like the bioactive polyphenols ellagitannins which exert antioxidant and anti-inflammatory effects. In aquaculture, studies have shown antibacterial, antiviral, anti-parasitic, immunostimulant and growth promoting activities (Pirbalouti et al. 2011; Harikrishnan et al. 2010a, Harikrishnan et al. 2010b). Bermuda grass (Poales, Poaceae) chemical composition includes phenolic compounds (gallic acid), tannins (catechins), anthocyanins (cyanidin) and flavonoids (quercetin) (Khlifi et al. 2013). *C. dactylon* displays antibacterial, antiviral, antiparasitic, immunostimulant and growth promoting activities in fish and shellfish (Balasubramanian et al. 2008a,b; Kaleeswaran et al. 2011). Indian ginseng (Solanales, Solanaceae) has been used for centuries in Ayurvedic medicine to increase longevity and vitality. Scientific research has indeed showed antioxidant, anti-inflammatory, immune-modulating, and anti-stress properties in the whole plant extract. *W. somnifera* biologically active chemical constituents include alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins) and saponins containing an additional acyl group (sitoindoside VII and VIII) (Mishra et al. 2000). *W. somnifera* showed to have antibacterial, antiviral, immunostimulant and growth promoting activities in aquaculture (Sharma et al. 2010; Yogeewaran et al. 2012; Talpur et al. 2013). Ginger (Zingiberales, Zingiberaceae) is an herbaceous perennial plant used for culinary and medicinal purposes. Ginger contains a mixture of zingerone, shogaols, and gingerols as well as some sesquiterpenoids, with (-)-zingiberene as the main component (Ali et al. 2008) that display bioactivities like antioxidant, anti-inflammatory, antibacterial and apoptosis inducer (Dugasani et al. 2010; El-Ghorab et al. 2010). In aquaculture, enrichment of diets with ginger has showed to promote growth and immunostimulate as well as display antibacterial, antiviral, antifungal and anti-parasitic activities (Nya and Austin, 2009; Caruana et al. 2012; Rajeswari et al. 2012; Kanani et al. 2014).

3.3. Analysis of plant bioactivities

Analysis of plant bioactivities showed that 36% of the plants studied presented antibacterial activity, whereas 17% had anti-parasitic activity, 16% immunostimulant activity, 14% antiviral activity, 13% growth promoter and only 4% showed antifungal activity (Figure 4). Nevertheless, it should be taken

into account, that those proportions are biased by the number of studies performed to date on each targeted pathology; for example many more studies have been focused on antibacterial activities than antifungal activities.

Plant bioactivities were also analysed by plant order and we found that Asterales was the order with the highest number of bioactive plants (34), with nearly half of them displaying antibacterial activity (Figure 5). Lamiales and Malpighiales were the two following orders with the highest number of bioactive plants (33 and 32). The Lamiales order had the highest number of plants displaying immunostimulant activity (7) and antifungal activity (5), while the Malpighiales presented the highest



antiviral activities (11) (Figure 5).

Figure 4. Plant bioactivities in aquaculture.

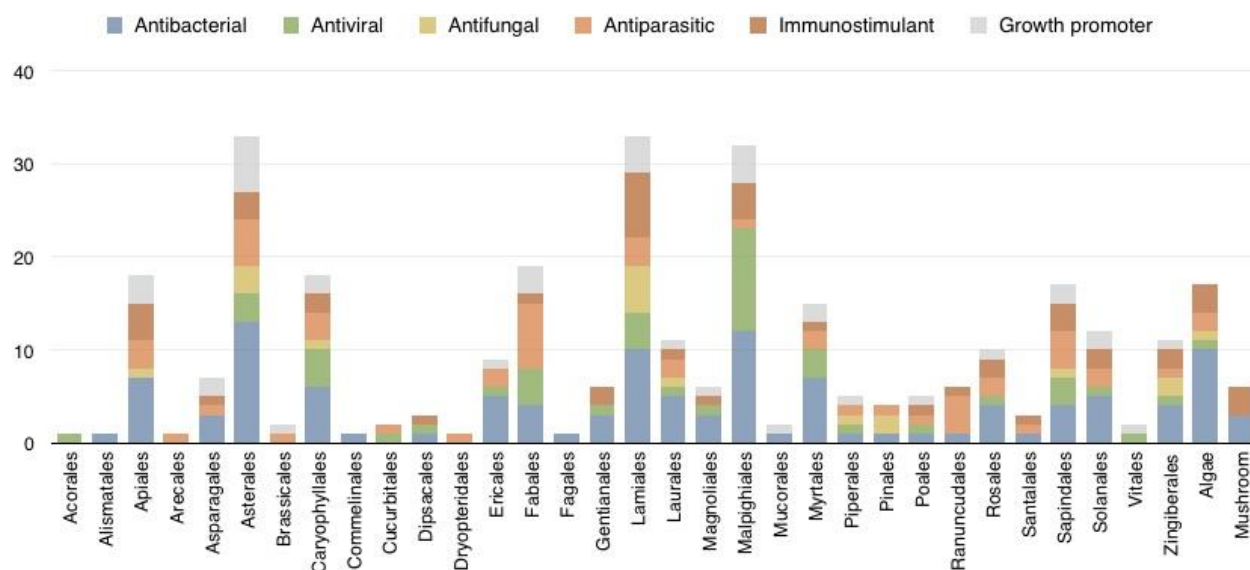


Figure 5. Plant order bioactivities in aquaculture.

3.4. Analysis on the plant parts used in aquaculture

Most of the studies on the use of medicinal plants in aquaculture used plant leaves (37%), while 22% of them used the whole plant either as powder, plant essential oil or extract. Root was also often used with (18%), followed by seeds (8%), barks (6%), fruits (6%) and finally flowers (4%) (Figure 6).

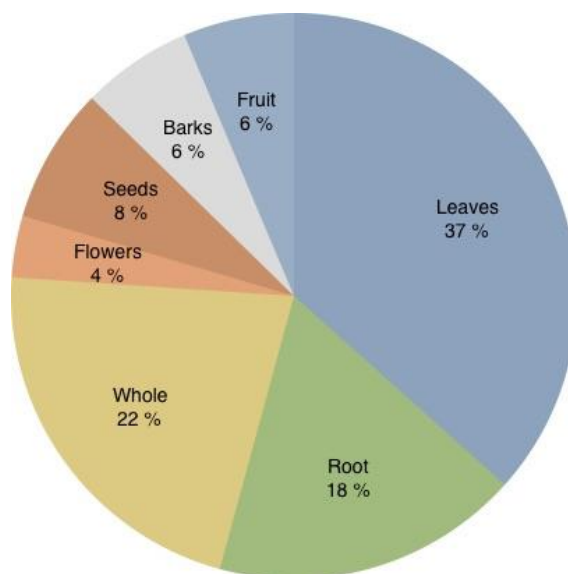


Figure 6. Parts of plants used in aquaculture.

3.5. Other plants and perspectives

Some algae and some mushrooms have also been studied for their potential application in aquaculture. Algae are considered to be a rich source of bioactive molecules, and most of the studied algae presented high antibacterial activities, and some also presented immunostimulant, antifungal, antiparasitic and antiviral activities (Choudhury et al. 2005; Hutson et al. 2012; Sukoso et al. 2012; Zbakh et al. 2012; Genovese et al. 2013). For example, red algae *Asparagopsis taxiformis*, which is known to produce a high diversity of halogenated metabolites, displayed antibacterial, antifungal and antiparasitic activities against several fish pathogens (Genovese et al. 2012, 2013). Also, *A. taxiformis* enhanced immune system of *Penaeus monodon* and was highly efficient in the treatment of Vibriosis in *P. monodon* (Manilal et al. 2012 ; 2013). Also, some studies like Mai et al. (2015) are starting to show interesting properties of other marine organisms like sponges in the quorum sensing inhibition of marine pathogenic bacteria such as *Vibrio harveyi*. Nevertheless, more research needs to be performed in this field, in order to establish the possibilities of application in aquaculture disease management.

Finally, there are numerous medicinal plants used in human medicine that have not yet been tested on aquaculture. For example, the gum rockrose (*Cistus ladanifer*, Cistaceae, Malvales) is a widely used

plant in several Mediterranean countries which possesses anti-inflammatory, antibacterial and antioxidant activities (Neves et al. 2009 ; Ferreira et al. 2012). Dandelions (*Taraxacum officinale*, Asteraceae, Asterales) have long been used in the Northern hemisphere for its diuretic, choleric, anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-hyperglycemic, anti-coagulatory and prebiotic effects (Schütz et al. 2006). Therefore, studies on the medicinal plants potentially used in aquaculture, should also explore the ethnobotanic studies in order to identify other interesting plants.

4. Conclusion

Medicinal plants (including algae and mushrooms) present promising potential to be used in aquaculture as a substitute of chemotherapy in the treatment of diseases outbreaks. Ethnobotanical studies have shown to be highly useful in the discovery of bioactive plants, and bioactive natural products with interesting applications in aquaculture. However, there is still scarce knowledge on the mode of action of most bioactive plants, as well as the most suitable form for an effective and safe administration. More research is needed to elucidate the plant natural products and their modes of action (establish the bioactive parts of the plant and the most suitable preparations), and to test the plant effects on the organisms physiology in order to establish an appropriate treatment (way of administration, dose and length). Besides, research works could also highly benefit from traditional knowledge of fish farmers who use regularly plants.

Finally, most studies have focused on the potential of terrestrial plants in aquaculture. Although, plants indeed offer interesting biological activities, they are not a natural component of neither the fish alimentation or the marine environment. Thus, continuous introduction of plant extracts and their bioactive compounds (through bath treatment or food diffusion) in the marine or freshwater environment, could eventually cause environmental problems. Therefore, the use of bioactive algae and seaweeds from the local natural environments where the aquaculture is performed, could represent a better alternative to avoid introduction of exogenous molecules into the environment. Algae are considered to be a rich source of original bioactive molecules which display multiple bioactivities. In aquaculture, several recent studies have showed the algae potential for the treatment of pathogens or to improve fish fitness, however they can also present undesirable effects and toxicity on fish, therefore more research needs to be performed on the physiological effects of algae on fish and the suitable dosing (Manilal et al. 2010).

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Résumé substantiel

Les parasites sont des organismes essentiels à la bonne santé des écosystèmes. Ils jouent un rôle vital dans les interactions entre espèces et contribuent à la sélection naturelle des espèces et à l'organisation des communautés. Les écosystèmes naturels supportent une grande diversité et richesse des parasites, qui contribuent à l'équilibre hôte-parasite. Néanmoins, dans certains cas comme dans l'aquaculture où les individus sont soumis à des stress liés au confinement et aux densités importantes, les interactions hôte-parasite sont modifiées, ce qui mène souvent à des épidémies et de grandes mortalités des hôtes.

Dans ma thèse j'ai étudié les interactions hôte-parasite chez les poissons de récifs coralliens, dans les milieux naturels et les milieux aquacoles. J'ai étudié l'arrangement des communautés de 13 espèces de monogènes de 34 espèces de poissons papillon (Chaetodontidae) dans l'Indo-Pacifique. Les résultats montrent un patron de distribution stable des espèces, avec différentes espèces de poisson papillon parasitées par différentes espèces de monogènes. L'étude biogéographique montre des différences prononcées entre les communautés de monogènes de Polynésie française, de Ningaloo Reef et du Pacifique Ouest (Lizard Island, Palau, Wallis & Futuna). Les différences entre les compositions taxonomiques de monogènes (turnover) sont corrélées avec la composition taxonomique des hôtes à chaque site, suggérant que la spécificité parasitaire observée a pu se développer tout au long des épisodes biogéographiques passés. Les différences entre les abondances relatives des espèces de monogènes (nestedness) sont corrélées avec des paramètres environnementaux, ce qui indique l'importance des conditions environnementales favorables pour l'éclosion des œufs des monogènes et pour l'infection efficace des hôtes.

L'étude épidémiologique a montré qu'une espèce de poisson papillon, *Chaetodon lunulatus*, n'est jamais parasitée, tandis que des espèces sympatriques qui présentent des écologies similaires et des positions phylogénétiques proches comme *Chaetodon ornatissimus* sont toujours et fortement parasitées. J'ai étudié le mucus des poissons, décrit comme la première surface d'échange entre le poisson et l'environnement tout en participant à la protection du poisson contre des pathogènes et à la communication avec d'autres organismes, pour analyser quels facteurs de *C. lunulatus* pourraient être reliés à l'absence des parasites.

L'étude du microbiome de quatre espèces de poisson papillon montre une grande diversité (indice de diversité de Shannon, $H = 3,7 - 5,7$). Les compositions bactériennes varient entre les différentes espèces, les microbiomes de *C. lunulatus* et *C. ornatissimus* étant les plus semblables, significativement différents des microbiomes de *C. vagabundus* and *C. reticulatus*. Les Proteobactéries apparaissent comme étant le taxon bactérien le plus abondant chez les quatre

espèces de poisson papillon. Les autres taxons présents chez les quatre espèces de Chaetodon incluent les Actinobactéries et les Firmicutes.

J'ai étudié le métabolome du mucus branchial de huit espèces de Chaetodon (*C. auriga*, *C. lunula*, *C. lunulatus*, *C. quadrimaculatus*, *C. ornatissimus*, *C. reticulatus*, *C. ulietensis* et *C. vagabundus*) avec des régimes alimentaires différents (corallivores facultatifs, corallivores obligatoires et omnivores), différentes positions phylogénétiques et différents taux de parasitisme branchial. Une extraction biphasique du mucus branchial des Chaetodons a permis d'obtenir un maximum de composés ; les deux fractions obtenues (polaire and apolaire) ont été étudiées en utilisant une approche métabolomique non-ciblée en chromatographie liquide couplée à la spectrométrie de masse (LC-MS). J'ai étudié la possible relation entre les différences métaboliques observées et les caractéristiques écologiques et phylogénétiques de chaque espèce. Le régime alimentaire est le paramètre qui explique la plus grande partie de la variabilité métabolique dans les deux fractions, tandis que le parasitisme est le deuxième facteur qui explique la variabilité métabolique de la fraction polaire.

J'ai ensuite intégré les résultats obtenus à partir du microbiome et du métabolome du mucus branchial des Chaetodon pour chercher s'il y a des taxons bactériens spécifiques et des métabolites surexprimés chez les poissons non parasités (*C. lunulatus*) qui pourraient être reliés à l'absence des parasites chez cette espèce. *C. lunulatus* présente une plus grande abondance des Fusobactéries et Spirochaetes qui sont fortement corrélées avec une surexpression de peptides qui pourraient provenir de la β -hémoglobine. Leur purification et leur séquençage sont en cours au laboratoire et leur synthèse sera réalisée pour pouvoir déterminer leur activité antiparasitaire.

Dans une deuxième partie de ma thèse, menée sur des poissons en élevage, j'ai étudié l'utilisation des plantes. Des études supplémentaires sont en cours au laboratoire pour identifier et caractériser les molécules antibactériennes d'*A. taxiformis*. De plus, d'autres études in vivo sont nécessaires pour évaluer la capacité réelle d'*A. taxiformis* contre des infections bactériennes en aquaculture. Les plantes médicinales comme alternative à des traitements chimiques en l'aquaculture. En effet, les plantes et les algues ont été utilisées dans l'aquaculture traditionnelle pendant longtemps. Plusieurs études ont montré leur capacité immunostimulante et leur activité contre nombreux pathogènes marins comme des bactéries, champignons, parasites et virus. De plus, elles présentent une alternative plus durable à l'utilisation des médicaments persistants qui sont aujourd'hui utilisés en grande quantité dans le traitement des infections aquacoles et qui présentent de nombreux inconvénients tels que le développement des bactéries résistantes, leur accumulation dans le tissu des animaux ou leur effet sur l'environnement. J'ai testé l'activité antibactérienne et immunostimulante de plusieurs plantes et algues polynésiennes. L'algue rouge *Asparagopsis taxiformis* montre de fortes activités antibactériennes sur *Vibrio harveyi* et *Tenacibaculum maritimum*, deux bactéries responsables de hautes mortalités chez de nombreuses

espèces de poissons. De plus, l'incorporation d'*A. taxiformis* dans le régime alimentaire des alevins du poisson chauve-souris (*Platax orbicularis*) induit une augmentation de l'expression de deux gènes liés à l'immunité (lysozyme G et TGF- β 1) chez *P. orbicularis*, ainsi qu'une augmentation de la croissance. Des études supplémentaires sont en cours au laboratoire pour identifier et caractériser les molécules antibactériennes d'*A. taxiformis*. De plus, d'autres études *in vivo* sont nécessaires pour évaluer la capacité réelle d'*A. taxiformis* contre des infections bactériennes en aquaculture.

En conclusion, les résultats de cette thèse montrent l'importance d'étudier les relations hôte-parasite par une approche globale. En effet, l'intégration des études épidémiologique, biogéographique, de l'analyse du métabolome et du microbiome, montrent la complexité des interactions poisson-parasite et l'importance de l'utilisation de différentes disciplines pour mieux comprendre leur fonctionnement. Les résultats même partiels amènent de nouvelles voies pour l'utilisation de produits naturels pour les traitements des pathologies en aquaculture.

Titre : Interactions hôte-parasite chez les poissons de récifs coralliens.

Résumé : Les parasites sont une partie très importante des écosystèmes, néanmoins, dans certains cas tels que l'aquaculture ils causent des épidémies. Dans cette thèse j'ai étudié les interactions hôte-parasite chez les poissons coralliens, dans les milieux naturels et les milieux aquacoles. J'ai étudié l'arrangement des communautés de 13 espèces de monogènes de 34 espèces de poissons papillon dans l'Indo-Pacifique. Les résultats montrent qu'il existe un patron de distribution stable des espèces, ce qui, combiné avec les résultats de l'étude biogéographique, suggère que la spécificité parasitaire observée a pu se développer tout au long des épisodes biogéographiques passés. Notamment, seule une espèce, *Chaetodon lunulatus*, n'est jamais parasitée. J'ai étudié le mucus des poissons qui est décrit comme la première ligne de défense contre des agressions externes, pour analyser quels facteurs de *C. lunulatus* pourraient être reliés à l'absence des parasites. Le microbiome et le métabolome du mucus branchial des poissons papillons montrent une grande diversité. *C. lunulatus* présente une plus grande abondance de Fusobactéries qui est corrélée avec une surexpression de peptides pouvant dériver de la β -hémoglobine. La purification, la synthèse et l'évaluation des activités antiparasitaires des peptides sont en cours au laboratoire. J'ai aussi étudié l'utilisation des plantes médicinales comme alternative à des traitements chimiques dans l'aquaculture. J'ai testé l'activité antibactérienne et immunostimulatrice de plusieurs plantes et algues polynésiennes. L'algue rouge *Asparagopsis taxiformis* montre de fortes activités antibactériennes sur *Vibrio harveyi* et *Tenacibaculum maritimum* et induit une augmentation de l'expression de deux gènes liés à l'immunité chez *Platax orbicularis*.

Mots-clés : interactions hôte-parasite, poissons coralliens, spécificité parasitaire, mucus de poisson, contrôle parasitaire, plantes médicinales.

Title: Host-parasite interactions in coral reef fish.

Abstract : Fish parasites are an important part of ecosystems, however, in certain cases such as in aquaculture they can cause severe disease outbreaks. In this thesis I have studied host-parasite interactions in coral reef fishes, both in the natural and culture environments. I have studied the distribution of 13 dactylogyrid species from 34 butterflyfishes in the Indo West-Pacific. Composition of dactylogyrid communities was host specific and together with the biogeography results, where a turnover in the main *Haliotrema* species was observed, suggest that parasite specificity might result from host-parasite coevolution derived from past biogeographical episodes. Only one butterflyfish species, *Chaetodon lunulatus*, was never found parasitized by gill monogeneans. I have studied the butterflyfish mucus, which is the first barrier against pathogens, to investigate the *C. lunulatus* factors that might be related to the monogenean absence. Butterflyfish gill microbiome and metabolome revealed a high diversity. *C. lunulatus* presented a significantly higher abundance of Fusobacteria which was correlated to a higher expression of potentially derived β -hemoglobin peptides. Synthesis and evaluation of the peptide antiparasitic activities are being performed in the laboratory. I have also studied the use of medicinal plants as an alternative to chemotherapy in fish aquaculture. I investigated the antibacterial and immunostimulant activities of several local Polynesian plants and algae, and I found that *Asparagopsis taxiformis* displayed a potent antibacterial activity against *Vibrio harveyi* and *Tenacibaculum maritimum* and increased expression of two immune-related genes in *Platax orbicularis*.

Keywords : host-parasite interactions, coral reef fish, parasite specificity, parasite evasion, fish mucus, parasite control, medicinal plants.