

**Tripartite symbiosis in *Hydra viridissima*:  
Understanding the interactions between  
host, algae and bacteria**

**Dissertation**

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## Project summary

The aim of the project was to understand the symbiotic interactions in *Hydra viridissima* metaorganism. The bipartite interaction between the host and the algae has been a subject of interest for decades, but the influence of bacteria on these interactions has always been overlooked.

The first part of the thesis focused on expanding the understanding of the metabolic co-dependence between the host and the algae. Presented in chapter 1, I showed the specificity of the metabolic genes in the host that aided in producing glutamine for the algae. Moreover, I could show that maltose, the photosynthetic sugar supplemented by the algae, was likely to be broken down into glucose before being utilized by the host.

In the second part of this thesis, I expanded the view on symbiotic interactions in *Hydra viridissima* by taking the bacterial colonizers into consideration. Chapter 2.1 focuses on understanding the effect of algal symbionts on the bacterial composition. I could demonstrate that co-cultivation of symbiotic (hosts with algae) and aposymbiotic (hosts without algae) animals resulted in the transfer of an invasive bacterium *Legionella sp.* to the symbiotic animals. This bacterium was fitness costly for the host; it reduced the growth rate of the host and increased algal expulsion. These expelled algae were further taken up by the aposymbiotic animals. However, under the influence of algal photosynthesis, this bacterium was eventually lost from the population, thus portraying the role of algae in controlling the invasive species and maintaining the core microbiome of the host.

Chapter 2.2 focuses on the effect of bacterial symbionts on the host and algal fitness. I assessed a wide array of fitness parameters and compared the germ-free (bacteria-free) symbiotic and aposymbiotic animals with the respective controls to study if there is any fitness loss in the absence of bacteria. Since the results were not conclusive and I could not observe any detectable effect of the bacteria, I replaced the native bacteria with complete sets of bacterial consortia from other *Hydra* species and assessed for their impact on host fitness. Interestingly, I could see reduced fitness in all animals that received foreign bacteria, thus portraying the importance of harboring the native, stable microbiota.

## Projektzusammenfassung

Das Ziel dieses Projektes war es, die symbiotischen Mechanismen im Metaorganismus *Hydra viridissima* zu verstehen. Während die Interaktionen zwischen dem Wirt und den Algen schon seit Jahrzehnten untersucht wird, wurde der Einfluss der Bakterien dabei bisher vernachlässigt.

Im ersten Teil dieser Arbeit habe ich mich auf die Untersuchung der gegenseitigen Abhängigkeit von Wirt und Algen fokussiert. Im ersten Kapitel konnte ich zeigen, wie die Spezifität der metabolischen Komponenten im Wirt dazu beitrug, Glutamin für die Algen zu produzieren. Weiterhin konnte ich zeigen, dass der photosynthetisch hergestellte Zucker der Algen, Maltose, wahrscheinlich in Glukose gespalten wird, bevor er vom Wirt weiter metabolisiert wird.

Im zweiten Teil dieser Arbeit habe ich den Blick auf die symbiotischen Interaktionen bei *Hydra viridissima* erweitert, indem ich die assoziierten Bakterien mit einbezog. Kapitel 2.1 behandelt den Effekt der symbiotischen Algen auf die Zusammensetzung des Mikrobioms. Ich konnte zeigen, dass die Kokultivierung von symbiotisch und aposymbiotischen Tieren zur Übertragung des invasiven Bakteriums *Legionella sp.* auf die symbiotischen Tiere führte. Dieses Bakterium reduzierte die Fitness des Wirts, indem es dessen Wachstumsrate verringerte und den Ausstoß von Algen erhöhte, welche dann von aposymbiotischen Tieren aufgenommen wurden. Allerdings verschwand dieses Bakterium unter dem Einfluss der Algenphotosynthese schließlich aus der Population. Dies verdeutlichte die Kontrolle durch die Algen auf die Invasion bakterieller Spezies und ihre Rolle in der Aufrechterhaltung des Kernmikrobioms des Wirts.

Kapitel 2.2 konzentriert sich auf den Effekt der bakteriellen Symbionten auf die Fitness des Wirts und der Algen. Ich untersuchte eine Vielzahl von Fitnessparametern zwischen keimfreien (bakterienfreien) symbiotischen und aposymbiotischen Tieren und den entsprechenden Kontrollen, um zu erfassen, ob es eine reduzierte Fitness in Abwesenheit der Bakterien gibt. Da die Ergebnisse nicht eindeutig waren und kein Effekt der Bakterien erkennbar war, ersetzte ich die natürlichen Bakterien mit bakteriellen Konsortien anderer *Hydra* Arten und untersuchte deren Einfluss auf die Wirt-Fitness. Interessanterweise konnte ich eine reduzierte Fitness in all diesen Tieren nachweisen, was die Wichtigkeit der art eigenen, stabilen Mikrobiota unterstreicht.

# 1 General introduction\*

Life has evolved in a microbial world. The event of successful symbiosis was a pivotal step that resulted in the origin of a eukaryotic cell. Organisms, therefore, are considered meta-organisms comprised of the macroscopic host and synergistic interdependence with bacteria, archaea, fungi, and numerous other microbial and eukaryotic species, including algal symbionts (Bosch & McFall-Ngai, 2011). The interactions between all the partners are affected by the prevailing environmental conditions. Understanding the mechanisms and the causality of the interactions in such a metaorganism requires model organisms where the contribution of each partner can be disentangled and functionally analyzed.

Aptly named, *Hydra* is known for its remarkable capacity of regeneration and non-senescence. For decades, *Hydra* has been used as a model organism to study the cellular pathways governing body patterning and aging by evolutionarily conserved mechanisms (Galliot, 2012). However, like all other metaorganisms, *Hydra* hosts numerous bacterial, viral, and algal symbionts (Fraune & Bosch, 2007; Grasis et al., 2014; Habetha, Anton-Erxleben, Neumann, & Bosch, 2003). For various reasons, *Hydra* turns out to be an excellent model system to study the interactions between these partners (Bosch, 2013). *Hydra* being diploblastic, possess a simple body plan composed of two stem cell epithelial layers- ectoderm and endoderm, separated by a thin layer of connective tissue, mesoglea. The third stem cell lineage, the interstitial cells, is localized in the ectoderm and gives rise to the nervous system, nematocytes, gland cells, and germline cells. Towards the outside on the ectoderm, a multilayered glycocalyx harbors the bacterial symbionts.

The extensive genomic and transcriptomic data of various *Hydra* species can be utilized to map known pathways and look for evolutionarily conserved genes. Moreover, it is also possible to genetically manipulate the host in loss/gain of function studies (Klimovich, Wittlieb, & Bosch, 2019; Wittlieb, Khalturin, Lohmann, Anton-

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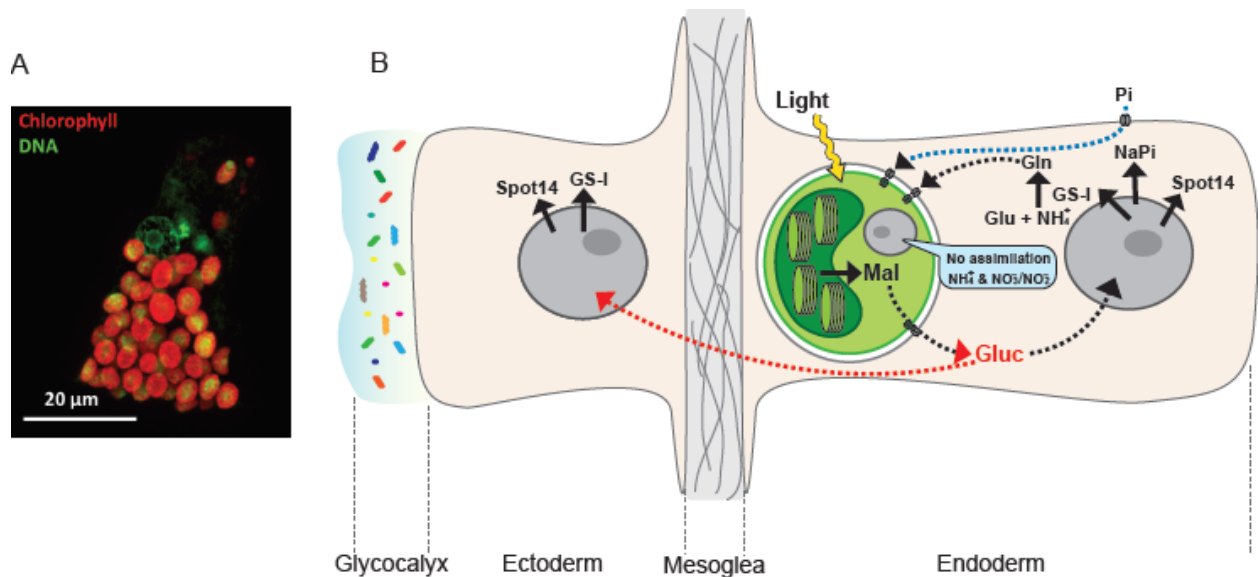
\* The introduction has been submitted as a book-chapter entitled, "Symbiotic interactions in the holobiont *Hydra*", authored by me and my supervisor, Thomas C.G. Bosch at CRC publishing house for a book entitled, "*Cellular Dialogues in the Holobiont*".

Erxleben, & Bosch, 2006). The transparent tissue facilitates the visualization of cellular components without rigorous fixing and bleaching methods. Large populations of *Hydra* can be easily cultivated and maintained under lab conditions. Clonal propagation ensures the genetic homogeneity of the entire population. In addition, since *Hydra* belongs to the phylum Cnidaria which is the sister group of Bilaterians, it is an informative model to study evolutionarily conserved mechanisms in metazoans. Being able to separate all the partners of the metaorganism makes *Hydra* suitable to explore the underlying principles of the complex multiplayer interactions ( Li et al. 2015; Li et al. 2017; Wein et al. 2018). This chapter summarizes the state-of-the-art understanding of the *Hydra* metaorganism, the interactions between the host and algal symbiont in *Hydra viridissima*, and also the interactions of the *Hydra* host with the bacterial symbionts.

## 1.1 Interactions between *Hydra viridissima* and the *Chlorella* photobiont

### Location and transmission of the photobiont

Symbiotic *Chlorella* algae are present intracellularly in the endodermal epithelial cells. This location points to a high level of co-dependence and rather complex cellular machinery involved in the maintenance of the symbiosis. Inside the *Hydra* endothelial cells, the algae are held towards the base of the cells (Jolley & Smith, 1978) (**Figure 1.1A**). This compartmentalizes an endodermal cell into two zones: digestive zone, which is the apical part of the cell responsible for phagocytosis and digestion of the food vesicles and the symbiotic zone, which is at the base of the cell where the symbiotic algae are present (McNeil, 1981; McNeil, Hohman, & Muscatine, 1981). Inside the host cell, each algal cell is surrounded by a host-derived vesicle membrane that acts as an interface for the biotrophic exchange of nutrients. This ‘peri-symbiotic’ membrane is the result of the phagocytosis by which the symbiont is taken up (Davy, Allemand, & Weis, 2012; McNeil, 1981).



**Figure 1.1: The *Hydra viridissima* - *Chlorella* symbiosis.**

(A) Each endothelial epithelial cell harbors about 30 *Chlorella* algae (red autofluorescence) in the basal part below the host nucleus (stained green). (Image courtesy: Jay Bathia and Alexander Klimovich, Zoological Institute, Kiel University) (B) Schematic summary of symbiotic interactions between *Hydra* and *Chlorella* A99. During light conditions, *Chlorella* A99 performs photosynthesis and produces maltose (Mal), which is secreted into the *Hydra* symbiosome where it is possibly digested to glucose (Gluc), shown in red. The sugar induces expression of *Hydra* genes encoding glutamine synthetase (GS), Na/Pi transporter (NaPi), and Spot14. GS catalyzes the condensation of glutamate (Glu) and ammonium ( $\text{NH}_4^+$ ) to form glutamine (Gln), which is used by *Chlorella* as a nitrogen source. Since the sugar also upregulates the NaPi gene, which controls intracellular phosphate levels, it might be involved in the supply of phosphorus to *Chlorella* as well (broken blue line). The sugar is also transported to the ectoderm (broken red line), and there induces the expression of GS and Spot14 (modified from Hamada et al. 2018).

The peri-symbiotic membrane is congruent to the membrane of a phagosome. The resulting structure is often referred to as a 'symbiosome' and the space between the peri-symbiotic membrane and the algae is called 'peri-symbiotic space' (Davy et al., 2012). Being present intracellularly demands a need for a successful transfer of the symbiont to the next generation. *Chlorella* algae can be transmitted horizontally as well as vertically. The host has a capacity to expel excess of living and preferentially dividing algae actively (Baghdasarian & Muscatine, 2000). Such algae can be later taken up from the environment by an aposymbiotic host to reestablish symbiosis. Vertical transmission takes place during the asexual reproduction phase because the endodermal cell layer of the mother polyp is in continuation with that of



the developing bud, which ensures the transfer of algae in the resulting bud (Jolley & Smith, 1980). Interestingly, symbiotic algae can also be transmitted vertically to the offspring through germ-line maternal cells (Campbell, 1990).

### **Mutual benefits**

The mutual exchange of metabolites between the host and the photobiont results in a phenotypic benefit for both the partners. The population growth rate of symbiotic animals is faster than aposymbiotic animals (Habetha et al., 2003; Hamada et al., 2018; Ishikawa et al., 2016). Moreover, the symbiotic animals can survive for a longer period under starvation as compared to the aposymbiotic animals (Ishikawa et al., 2016). It has also been observed that the presence of algae increases the frequency of host oogenesis (Habetha et al., 2003). Since oogenesis is typically an energetically expensive process, the transport of sugar from the algae may aid in overcoming the cost of oogenesis. Interestingly, any attempt to culture the endosymbiotic algae *in vitro* consistently failed. This underlines the strong dependence of the algae on the host. Apart from being nutritionally beneficial, the algae can protect the host under environmental stress. A recent study (Ye, Badhiwala, Robinson, Cho, & Siemann, 2019) indicates that if symbiotic algae were acclimated to higher temperatures, they provide survival benefits to the *Hydra* holobiont at higher temperatures. Several studies utilized the ability of *Hydra viridissima* to establish symbiosis with foreign endosymbiotic algae such as *Chlorella* NC64A (Dorling, Mcauley, & Hodge, 1997; Hamada et al., 2018; Kodama & Fujishima, 2015). *Chlorella* NC64A is a native endosymbiont of the protist *Paramecium bursaria*. *Chlorella* NC64A can release sugar to the host only in low amounts, resulting in a lower population growth rate, almost equivalent to aposymbiotic animals (Hamada et al., 2018), compared to polyps harboring the native photobiont *Chlorella* A99. This further indicates the importance of harboring a native symbiont.

### **Establishment and maintenance of the *Chlorella-Hydra* symbiosis**

To establish a new symbiosis, the aposymbiotic host engulfs the algae through phagocytosis. This results in the formation of a phagosome enclosing the

algae. It is acidified by the fusion with acidosomes (Allen & Fok, 1983). Only if the engulfed algae is a true symbiont, the fusion of lysosome is inhibited, indicated by the absence of acid phosphatase activity in the late phagosome stage (Hohman, Mcneil, & Muscatine, 1982). The recognition of symbiotic algae occurs probably by the presence of Microbe associated molecular patterns (MAMPs). Recent comparative transcriptomic studies indicate the putative involvement of the Toll/Interleukin-1 receptor (TIR), a known Pattern recognition receptor (PRR) and C-type lectin, to be involved in recognizing the MAMPs of symbiotic algae (Hamada et al., 2018).

The physiological conditions and, in particular, the pH experienced by an endosymbiotic alga are very different from a free-living alga. The pH of the peri-symbiotic space is maintained between 4 and 5. This is crucial because only at this low pH, the algae is shown to be able to release maltose (Cernichiari, Muscatine, & Smith, 1969; Dorling et al., 1997). The maltose releasing capacity is unique to endosymbiotic algae. In plants and free-living algae, during nighttime, the stored starch in chloroplasts is mobilized to the cytoplasm in the form of maltose, but never secreted to the extracellular space (Lu & Sharkey, 2006). This may point to maltose transport machinery on the membrane of the symbiotic algae similar to the membrane of the chloroplast. Another important characteristic of a successful mutualistic relationship is to control the proliferation of the endosymbiont. There is evidence that *Hydra* controls the number of algae per cell by regulating the pH of the peri-symbiotic space (Dunn, 1987; McAuley, 1986; L. Muscatine & Neckelmann, 1981). Moreover, the host is capable to actively expel any excess number of algae (Baghdasarian & Muscatine, 2000).

### **Molecular mechanisms involved in maintaining the symbiosis**

An elaborated comparative transcriptomics and genomic study of *Hydra viridissima* revealed the key components and pathways involved in maintaining the host-algal symbiosis (Hamada et al., 2018). To identify the host genes involved in maintaining symbiosis with the native algae, the unbiased high-throughput study (Hamada et al., 2018) included a comparison of the symbiotic host associated with

the native symbiotic *Chlorella* A99, the aposymbiotic host, and the host bearing foreign symbiotic algae *Chlorella* NC64A.

Comparing the native symbiotic animals with the aposymbiotic animals revealed the upregulation of a number of conserved cellular pathways in symbiotic animals that maintain the symbiosis. The transcriptomics data revealed the upregulation of a host V-type ATPase subunit and carbonic anhydrase, involved in maintaining the low pH of the perisymbiotic space and facilitating CO<sub>2</sub> transport across the symbiosome respectively. Moreover, there is an upregulation of GLUT8-like transporters, putatively involved in the transportation of the photosynthate across the peri-symbiotic membrane. The study also uncovered the up-regulation of cell-adhesion genes encoding rhamnospondin and fibrillin. These molecules might be involved in retaining the algae to the base of the endothelial cell. Moreover, a high level of peroxidase, methionine-r-sulfoxide reductase/selenoprotein, and glutaredoxin might help to combat the surge in oxidative stress generated by the constant oxygen influx from the algae to the host.

A comparison of *Hydra* polyps harboring *Chlorella* A99 with *Hydra* polyps colonized by the non-native *Chlorella* NC64A algae showed that the interactions between the host and the algae are mainly metabolic in nature (summarized in **Figure 1.1B**). The native photobiont provides the photosynthetically fixed carbon to the host in the form of maltose. This maltose is likely to be digested to its monomer glucose, which is transported to the host by transporter proteins in the symbiosome membrane. This excess sugar shifts the host metabolism to ammonium assimilation. In response to this, there is an upregulation of host glutamine synthetase (GS-I), which is involved in the production of glutamine from ammonium and glutamate. GS-I is specific to the symbiotic state, as other isoforms are unaffected by the presence or absence of the algae. As another important stabilizer of the *Hydra-Chlorella* symbiosis, the sodium-dependent phosphate transport protein (NaPi) can increase the available phosphate for the host and the algae. Apart from these, a Spot\_14 like domain-containing protein with a potential role in lipid metabolism, and two taxonomically restricted genes (TRGs) are also upregulated. Exposing polyps to the photosynthesis inhibitor DCMU or culturing them at constant darkness results in a drastically reduced expression level of these genes, indicating that the expression of

these symbiosis-specific genes is dependent on the photosynthetic activity of the symbiont.

Genome analysis of the symbiotic *Chlorella A99* algae and the comparison to the genome of the free-living counterparts revealed interesting features that helped to explain the strong dependence of the symbiont on the host (Hamada et al., 2018). Most strikingly, the symbiotic algae have lost their key nitrite/nitrate and ammonium assimilation genes. Symbiotic algae, therefore, depend on the host for fixed nitrogen in the form of glutamine. Further genomic analyses of the algae also indicated an increase in the number of amino acid transporters, supporting amino acid uptake. Finally, although all attempts to culture these algae *in vitro* failed, the growth of the algae can be temporarily maintained by adding CAS amino acids or glutamine as a source of nitrogen to the culture medium. This reflects the strong metabolic co-dependence of both partners and a unique adaptation of the symbiotic algae (Hamada et al., 2018).

## 1.2 Interactions between *Hydra* and symbiotic bacteria

In addition to host-photobiont interactions in *Hydra viridissima*, all *Hydra* species are stably associated with a species-specific microbiome. Even after over 30 years of culturing in the lab under artificial conditions, *Hydra* species maintain their distinct bacterial composition, which resembled the microbiota of polyps freshly collected from the wild (Fraune and Bosch 2007; Franzenburg et al, 2013). This, together with the cellular and molecular accessibility of the *Hydra* polyp for functional approaches, allows investigating the interaction between microbes and *Hydra* polyps in an unprecedented manner and, at the same time, provides insight into the evolution of host-microbe interactions.

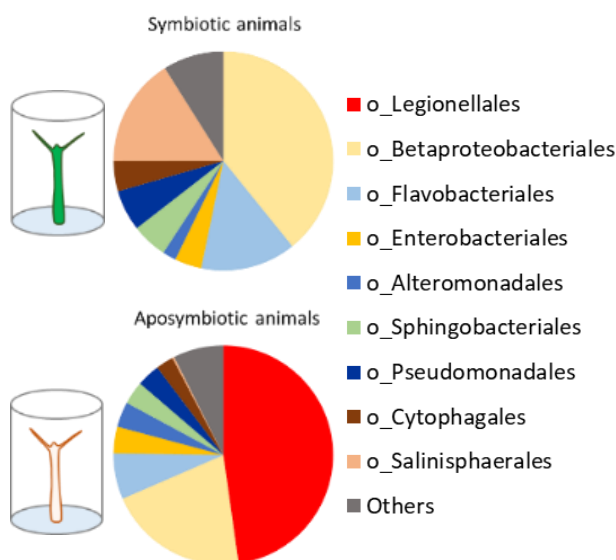
### **Spatial localization of the bacteria in the *Hydra* host**

The *Hydra* ectodermal epithelium can be considered as an inside-out gut, with the mucosal layer facing towards the outside (Schröder & Bosch, 2016). A

carbohydrate and glycoprotein rich layer called glycocalyx covers the outer surface of the polyp (**Figure 1.3**). The glycocalyx is a multi-layered structure composed of at least five layers, with the first four layers anchored to the ectoderm. Interestingly, the presence of bacteria was detected only in the most distal layer of the glycocalyx (Fraune et al., 2015), which acts as the first barrier between the environmental bacteria and the host tissue. In *H. oligactis*, a few of the bacterial symbionts are endosymbiotic in the ectodermal epithelial cells (Fraune & Bosch, 2007). Most of the functional studies were done in *H. vulgaris* AEP or *H. magnipapillata*, which do not harbor any endosymbiotic bacteria (Fraune et al., 2015; Fraune & Bosch, 2007).

### Microbiome composition of symbiotic and aposymbiotic *H. viridissima*

Like other *Hydra* species, green *Hydra* harbors a species-specific microbiome. Interestingly the microbiome composition of the symbiotic and aposymbiotic animals is different (**Figure 1.2**). At the order level, the microbiome of the symbiotic animals is dominated by the *Betaproteobacteriales* and by *Legionellales* in aposymbiotic animals. There is one dominant OTU in the aposymbiotic animals that belongs to the genus *Legionella*, while it is present in a very low abundance in the symbiotic animals. Since these aposymbiotic animals were generated from the symbiotic animals, it is likely that the absence of the algae was responsible for the observed difference in the microbiome composition (Bathia et al., in prep).



**Figure 1.2: Microbiome composition of the symbiotic and aposymbiotic animals**

The pie-charts show the average microbiome composition of the symbiotic and aposymbiotic animals. The classification at order level already reveals the microbiome composition difference between the symbiotic and aposymbiotic animals. The pie charts are the results of average microbiome composition of 6 biological replicates each. (Bathia et al., in prep.)

## **Bacteria provide protection against fungal infection**

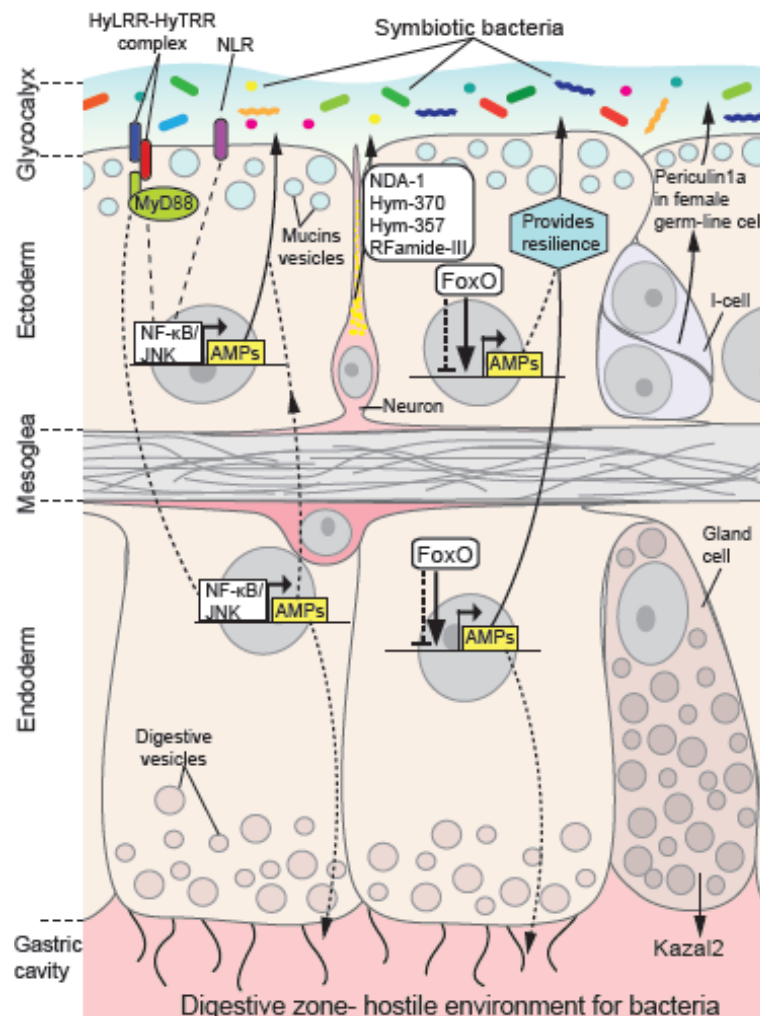
Being able to separate the *Hydra* host and bacterial partners provides an efficient analytical framework to disentangle the contribution of each partner to the working of the holobiont. The germ-free *H. vulgaris* AEP hosts are highly susceptible to the infection by *Fusarium* sp. fungus, suggesting a strong protective role of bacteria against a fungal pathogen. Colonization of hosts with single bacterial symbiont species failed to provide resistance against the fungi pointing to the importance of a complete and diverse bacterial community in maintaining the holobiont homeostasis (Fraune et al., 2015).

## **The innate immune system shapes the host microbiome**

Since the epithelial layer of *Hydra* is constantly in contact with a large variety of bacteria, including pathogens, a wide array of competing forces seems to ensure the selection of specific bacterial species from the pool of bacterioplankton (Deines, Lachnit, & Bosch, 2017). But do *Hydra* polyps actively select their microbial symbionts? On testing neutrality in the microbial composition for *Hydra vulgaris* AEP, it was recently observed that the microbiome composition deviates from the theoretically predicted neutral community, indicating that under the prevailing environmental conditions, a major part of the microbiota is affected by host-derived factors (Sieber et al., 2019).

These theoretical considerations are supported by experimental work, which over the last decade has shown that *Hydra*'s innate immune system plays a major role in shaping the species-specific microbiota (Bosch, 2014; Klimovich & Bosch, 2018). The recognition of the microbe-associated molecular patterns (MAMPs) occurs by Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD) like receptors (NLRs) (Bosch et al., 2009). The structure of *Hydra* TLRs is unconventional; it is composed of leucine-rich repeat (LRR) domain-containing protein (HyLRR) and a separate Toll/interleukin-1 receptor (TIR) domain-containing protein (HyTRR). Both these proteins interact to form a functional TLR. The TIR domain of HyTRR interacts with the TIR domain of myeloid differentiation factor-88 (MyD88) protein. Upon stimulation by MAMPs, the signaling finally

activates c-Jun N-terminal kinase (JNK) and *Hydra* orthologue of nuclear factor kappa-light-chain-enhancer of activated B cells (HyNF- $\kappa$ B). These transcription factors induce the expression of various anti-microbial peptides (AMPs) that play a crucial role in shaping the host microbiome (Augustin et al 2009; Bosch 2014).



**Figure 1.3: Summary of interactions between *Hydra* and symbiotic bacteria.**

Epithelial cells produce anti-microbial peptides (AMPs) belonging to Arminin/ Hydramacin/ Periculin families. Gland cells in the endoderm express Kazal family AMPs. I-cells, after being differentiated in to female germ-line cells express AMP-Periculin1a. Anti-bacterial neuropeptides NDA-1, Hym-370, Hym-357, and the RFamide III family are expressed in nerve cells. Stem-cell transcription factor FoxO plays a regulatory role in the expression of Hydramacin/ Arminin/ Kazal, without affecting Periculin and NDA-1. Mucin containing vesicles (in blue) in ectodermal epithelial cells contribute to the establishment of the glycocalyx. (Bathia and Bosch, in press)

Endodermal epithelial cells express the AMP peptide family “Hydramacin” (Bosch et al., 2009; Jung et al., 2009). Hydramacin expression is induced by MAMPs via the TLR signaling pathway and has antimicrobial activity against various gram-positive and gram-negative bacteria. The endothelial gland cells in the body column express a considerably high amount of Kazal2, a kazal-type serine protease inhibitor that targets the bacterial serine proteases (Augustin, Siebert, & Bosch, 2009) (Figure 1.3) and provides protection against pathogenic bacteria in the gastric cavity. Although *Hydra* lacks any migratory phagocytic cells, it does possess phagocytic activity in the endodermal cell. It is through this property, that the aposymbiotic *H.*

*viridissima* is able to establish a symbiosis with *Chlorella* algae. The same phagocytic property can also help to keep the endoderm free of bacteria by engulfing any bacteria entering with the food (Bosch et al., 2009).

Maternal protection of the developing progeny against pathogens or bacterial overgrowth is provided by the AMP Periculin1a, which is expressed exclusively in maternal germline cells (Fraune et al., 2010). This AMP persists during the initial phases of embryogenesis up to the blastula stage and controls the bacterial load as well as assists in the selection of the colonizing bacteria. The function of maternal Periculin1a is later taken over by zygotic Periculin2b after the mid-blastula transition. In an adult *H. vulgaris* AEP polyp, the endodermal knockdown of another family of AMP, the Arminin peptides, results in a reduced selection by the host for the colonizing bacterial partners. Since in the absence of Arminin peptides the bacteria from other donor species, *H. oligactis* and *H. viridissima*, can colonize the host, these AMPs are involved in maintaining a specific-specific microbiota.

The colonization of a newly hatched polyp follows a robust temporal trajectory (Franzenburg, Fraune, et al., 2013). There is high variability in the microbiota initially, followed by a transient increase in the relative abundance of the main colonizers of the adult microbiota. Following this, there is a drastic decrease in microbial diversity before it stabilizes to adult microbiota at the end of four weeks.

### **Crosstalk between innate immunity and stem cell factors**

In *Hydra*, and most likely in other organisms as well, the innate immune system is tightly linked to pathways controlling epithelial cell homeostasis. *Hydra*'s Forkhead box transcription factor (FoxO) is key to maintaining the stem cell identity of the ecto- and endodermal epithelial cells (Boehm et al., 2012) (**Figure 1.3**). The expression of FoxO is limited to the stem cell zone and absent in the terminally differentiated cells. Epithelial FoxO loss-of-function mutants revealed that a deficiency in FoxO signaling leads not only to malfunctions in cell cycle progression but also to dysregulation of multiple families of genes encoding antimicrobial peptides (AMPs). FoxO loss-of-function polyps were more susceptible to colonization by foreign bacteria and impaired in selection for bacteria resembling the native



microbiome. FoxO-deficiency reduces the expression of AMPs, resulting in decreased selective pressure on colonizing microbial taxa and, ultimately, in reduced resilience of the microbiome (Boehm et al., 2012; Mortzfeld, Taubenheim, Fraune, Klimovich, & Bosch, 2018).

### **Crosstalk between the microbiota and the nervous system**

In addition to the epithelial cells, the nervous system also plays an important role in shaping the microbial community of *Hydra*. The first indication of crosstalk between both the systems came from the observation that loss of nerve-cell lineage resulted in the increased anti-bacterial activity of the *Hydra* polyps (Fraune, Abe, & Bosch, 2009). A recent study (Augustin et al., 2017) provided evidence that some sensory and ganglion neurons express a cationic neuropeptide called NDA-1, secrete it into the mucus layer, and regulate the spatial distribution of the main colonizer, the Gram-negative bacterium *Curvibacter*, along the *Hydra* trunk. The density of *Curvibacter* colonization is relatively low in the foot and tentacles of *Hydra*, where NDA-1 is strongly expressed, compared to the body column. Additionally, NDA-1 is highly potent in killing Gram-positive bacteria. Strikingly, other neuropeptides, such as *Hydra* specific Hym-357 and Hym-370 and a member of the highly conserved RFamide family, all previously characterized as classical neuromodulators eliciting motor activity, turned out to be also potent against Gram-positive bacteria. Taken together, these findings indicate that distinct nerve cells contribute to the composition and spatial structure of *Hydra*'s microbial community by expressing a variety of neuropeptides with distinct antimicrobial activities.

Based on these observations, it was proposed (Klimovich & Bosch, 2018) that during evolution, the nervous system, in addition to its role in sensory input/motor output, plays a primordial role as part of the innate immune system.

All these observations, taken together, portray a rather complex network of molecular and cellular interactions controlling the establishment and maintenance of a stable microbiota in *Hydra* (**Figure 1.3**).

The level of complexity of the interspecies crosstalk in the holobiont *Hydra* is even larger since many of the bacterial partners harbor temperate phages bearing the capacity to cross infect other bacterial species. One such example is observed as competition between the top two main colonizers of *H. vulgaris* AEP, *Curvibacter* sp., and *Duganella* sp. (Li et al. 2015; Li et al. 2017). Associated with host, *Curvibacter* out-competes *Duganella*, while *in-vitro*, in monocultures, the effect is vice-versa. However, the co-culture of both the bacteria *in-vitro* results in a non-linear reduction of growth of *Duganella*, depending on the initial frequency of *Curvibacter*. When modeled mathematically, the interaction between both the bacteria in co-culture could only be explained by the presence of a third partner, a bacteriophage. Indeed, *Curvibacter* inhabits a pro-phage that can be activated to enter the lytic cycle and have lytic activity against *Duganella*. Thus, the *in-vivo* competition between the top two colonizers is likely to be mediated by a bacteriophage.

### **Effect of bacteria on host physiology**

With the host factors having a profound effect on its microbiome, there also exists a strong impact of the bacterial partners on the host physiology and host behavior. *Hydra* exhibits various behaviors like spontaneous contractions, feeding response to a stimulus, and phototactic movement, to name a few. Spontaneous contractions are assumed to be regulated by the pacemaker activity of the neurons (Passano and Mccullough 1965). Interestingly, the frequency of the spontaneous contractions in germ-free *H. vulgaris* AEP animals is reduced to ~60% of the control animals (Murillo-Rincon et al., 2017). This effect can be restored, although not completely, by recolonization of these germ-free animals by the normal microbiome. Moreover, the microbial extract from the *in-vitro* grown complete microbial community on a complex media resulted in the restoration of the contraction frequency largely. Bacteria or bacteria-derived molecules, therefore, directly interfere with neuronal activity and function.

## ***Hydra*, an excellent model to understand inter-species interactions**

Inter-species interactions in the freshwater polyp *Hydra* between symbiotic algae and host cells had been the subject of research for decades since they not only provide insights into the basic "tool kit" necessary to establish symbiotic interactions but are also of relevance in understanding the resulting evolutionary selection processes. A long term persistence of symbiotic associations is prevalent not only in two-party interactions of *Hydra* and symbiotic algae but also in more complex systems including stably associated bacteria and a species-specific virome (Franzenburg, Walter, et al., 2013; Grasis et al., 2014). Studying symbiotic inter-species interactions in *Hydra*, therefore, may be a paradigmatic example of a complex symbiotic community that influences the host's health and development. The results have contributed to a paradigm shift in evolutionary immunology: components of the innate immune system with its host-specific antimicrobial peptides appear to have evolved in early-branching metazoans because of the need to control the resident beneficial microbes rather than because of invasive pathogens.

## 2 Results

### 2.1 Chapter 1: Understanding metabolic co-dependence between the host and the algae

#### 2.1.1 Introduction

The interaction between the host and the algae is mainly metabolic in nature (Hamada et al., 2018). To dwell further into the molecular players involved in maintaining the homeostasis of symbiosis, a comparative transcriptomic experiment was performed, published as Hamada et al. 2018, eLife. I made a small contribution to understand these interactions and develop the current metabolic model of interactions, as mentioned in this chapter.

As introduced in the previous chapter, the elaborated comparative transcriptomic study involved a comparison between symbiotic animals (with *Chlorella A99*), aposymbiotic animals (animals without algae), and foreign symbiotic animals (same animals with *Chlorella NC64A*). This resulted in a list of 10 genes that were differentially expressed only in the presence of the native symbiotic algae and not in the presence of foreign symbiotic algae (**Table 2.1.1**). The upregulated genes were further tested and shown to be responding in aposymbiotic animals to the sugar secreted by the symbiotic algae, maltose, in a dose-dependent manner. However, the taxonomically restricted genes (TRGs) didn't show any effect of maltose treatment, indicating a more sophisticated mode of regulation (Hamada et al., 2018). Moreover, the transcriptomic study revealed that only one of the three host's glutamine synthetases is differentially expressed in the presence of the native symbiont.

### 2.1.2 Objective

The objective of this chapter was to study if the upregulated metabolic genes were broadly responsive to a variety of sugars, or it is specific to maltose in aposymbiotic animals. This would show if the response from the host is a generic metabolic response or specific to maltose and its breakdown product, glucose. Moreover, *H. viridissima* host has three glutamine synthetase isoforms, and the transcriptomics data showed only one of them responding to the presence of the native symbiont. So, it was intriguing to assess if other glutamine synthetases respond to other sugars as well. Furthermore, assaying the rapidity of the response becomes interesting because the immediate response to the sugars would distinctly show the signaling or metabolite nature of the sugar.

### 2.1.3 Methodology

The chapter focuses on investigating the effect of sugars on the metabolic genes involved in symbiosis. The aposymbiotic animals were treated with four different sugars- maltose, sucrose, galactose, and glucose at different concentrations- control, 10, 25, 50, 75, and 100 mM concentrations in 3 biological replicates each. The sugar solution was replaced after 24 hours with a fresh one. RNA was isolated after 48 hours from each treatment replicate from a pool of ~40 animals. 100 mM concentrations, being detrimental for the host, the samples were not used for further analysis. The detailed procedures are described in the chapter - **Materials and methods**. qRT-PCR measurements were normalized using actin, elongation-factor-1-a, and tubulin. The upregulated symbiosis genes (**Table 2.1.1**) except the taxonomically restricted genes (TRGs) and glutamine synthetase-2/3 were studied for the effect of the sugars using custom primers as described in **Supplementary Table 1**. A similar experiment was performed using maltose and glucose at 75 mM treatment concentration to observe the rapidity of response. For this, the aposymbiotic animals were treated with sugars for 3 hours and 6 hours prior to RNA isolation and assaying the levels of these genes using qRT-PCR. The data is

represented as a relative expression or log<sub>2</sub> fold-change with respect to controls. The relative expression refers to the ratio of treatment to control.

## 2.1.4 Results

### The response of the symbiosis genes to different sugars

Maltose is a dimer of glucose, and the breakdown of each maltose molecule would result in two glucose molecules. Therefore, the response of these genes was observed in the aposymbiotic animals to glucose and maltose. Moreover, as additional controls, galactose and sucrose were used as monomer and dimer sugar controls, respectively. It was astonishing to observe that in the preliminary experiment performed by former students, the aposymbiotic animals could survive the 100 mM maltose concentrations (Hamada et al., 2018); while in my experiments, the animals could not survive 100 mM maltose concentrations and 75 mM sugar concentration was used as maximum concentration. But, only in galactose, could the animals survive the 100 mM concentration (**Figure 2.1.1**).

Up-regulated gene	Down-regulated gene
Glutamine synthetase (GS-1)	Acetyl-CoA synthetase
Sodium-dependent phosphate transport protein (NaPi)	2-isopropylmalate synthase
Spot-14 like domain-containing protein (Spot-14)	Histidine ammonia-lyase
Hvir TRG-1 (13579)	Hvir TRG-3 (NPNHRC_26859)
Hvir TRG-2 (12891)	Hvir TRG-4 (NPNHRC_12201)

**Table 2.1.1: List of differentially expressed genes**

The table represents the list of 10 genes that were differentially expressed only in the presence of the native symbiont *Chlorella A99*. The comparison was made between the symbiotic animals (with native symbiont), aposymbiotic animals (no symbiont), and symbiotic NC64 animals (aposymbiotic animals containing *Chlorella NC64*). The expression of these genes was also confirmed by qRT-PCR.

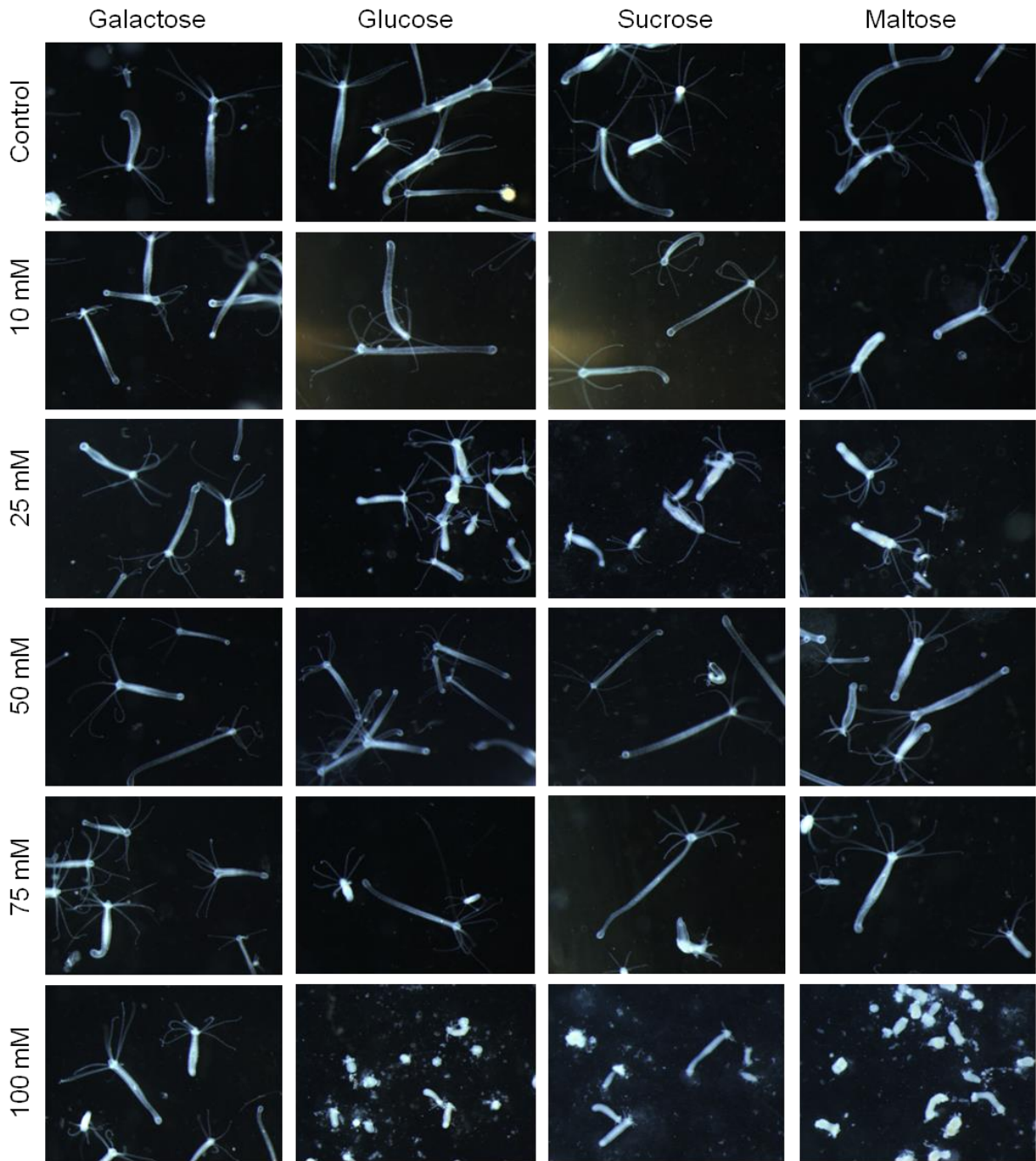
The animals were treated with sugars for 48 hours before RNA isolation and maintained with the 12h-12h diel cycle. qRT-PCR results showed that there was a concentration-dependent increase in the expression of the upregulated metabolic genes (from **Table 2.1.1**) when treated with maltose or glucose. There was a minimal effect of sucrose and galactose on these genes. In some cases, galactose showed a dose-dependent reduction in the expression because alpha-galactosidase can show substrate inhibition with increasing concentration (Liao et al., 2016) (**Figure 2.1.3 A-D**). This experiment was performed with 48 hours of sugar treatment. I further exposed the aposymbiotic animals with maltose and glucose for 3 hours and 6 hours to check the rapidity of the response. Surprisingly, these metabolic genes responded to the sugars even after 3 hours of treatment (**Figure 2.1.2 E-F**).

These results suggest the utilization of maltose as a metabolite and not a signaling molecule, which is likely to be digested to glucose and being used as a source of energy. However, the rapidity of maltose utilization came to be a real surprise.

### **Specificity towards Glutamine synthetase**

Comparative transcriptomics and genomic data revealed the occurrence of 3 isoforms of glutamine synthetase, later designated as GS-1, GS-2, and GS-3. It was also observed that only GS-1 was differentially expressed in the symbiotic animals.

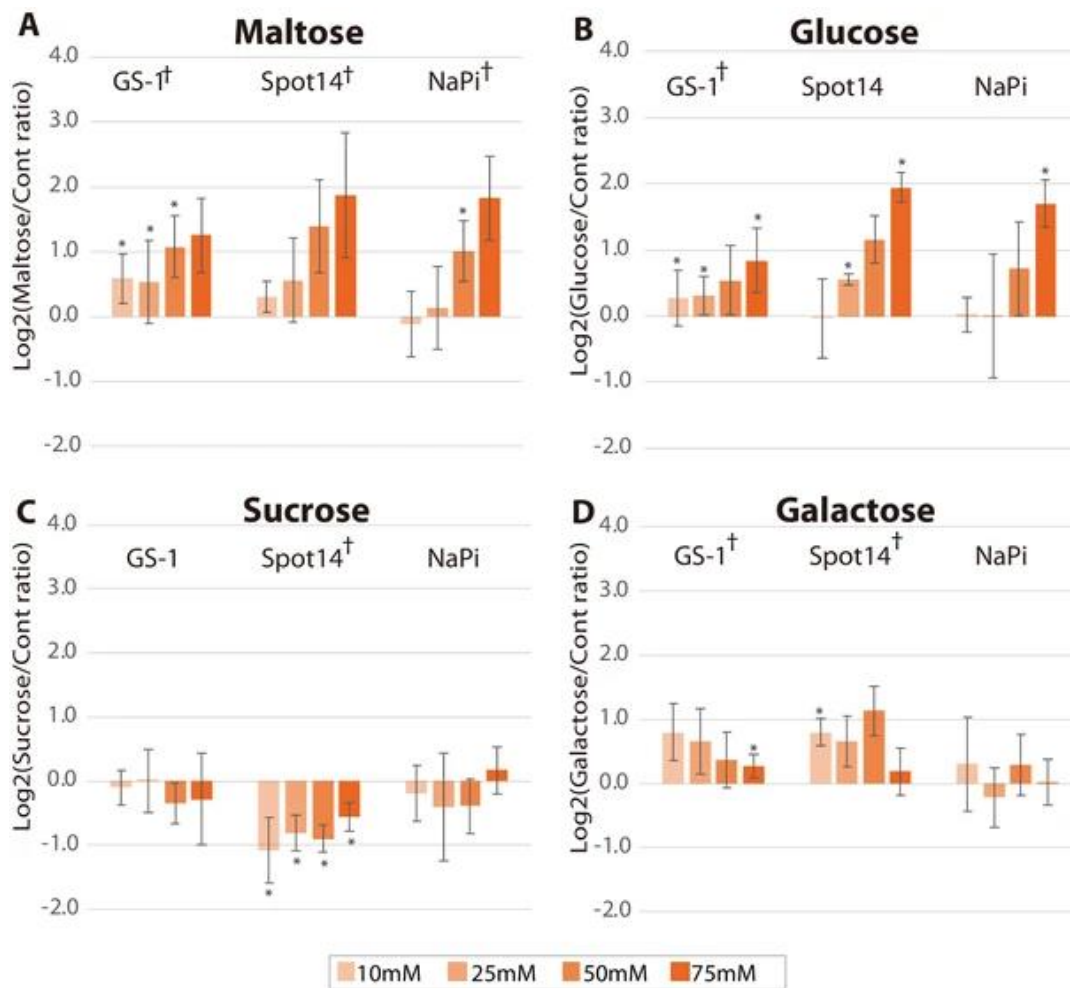
I later confirmed by qRT-PCR that only GS-1 responds to glucose and maltose treatment in aposymbiotic animals and can be induced only after 3 hours of maltose/glucose treatment (**Figure 2.1.3A-E**). These results were suggestive of the specificity of the algal secreted metabolites for the symbiosis specific glutamine synthetase. Thus, it is highly likely that GS-1 is responsible for the production of glutamine that is the primary source of nitrogen for the algal symbionts.



**Figure 2.1.1: Effect of different sugars on the fitness of aposymbiotic animals**

The aposymbiotic animals were treated with 0, 10, 25, 50, 75, and 100 mM of galactose/glucose/sucrose/maltose for RNA isolation and further analysis by qRT PCR of the target genes. Phenotypic observations suggested that except 100 mM galactose, other sugars of the same concentration were detrimental for the polyps. So, those detrimental samples were not used for RNA isolation.





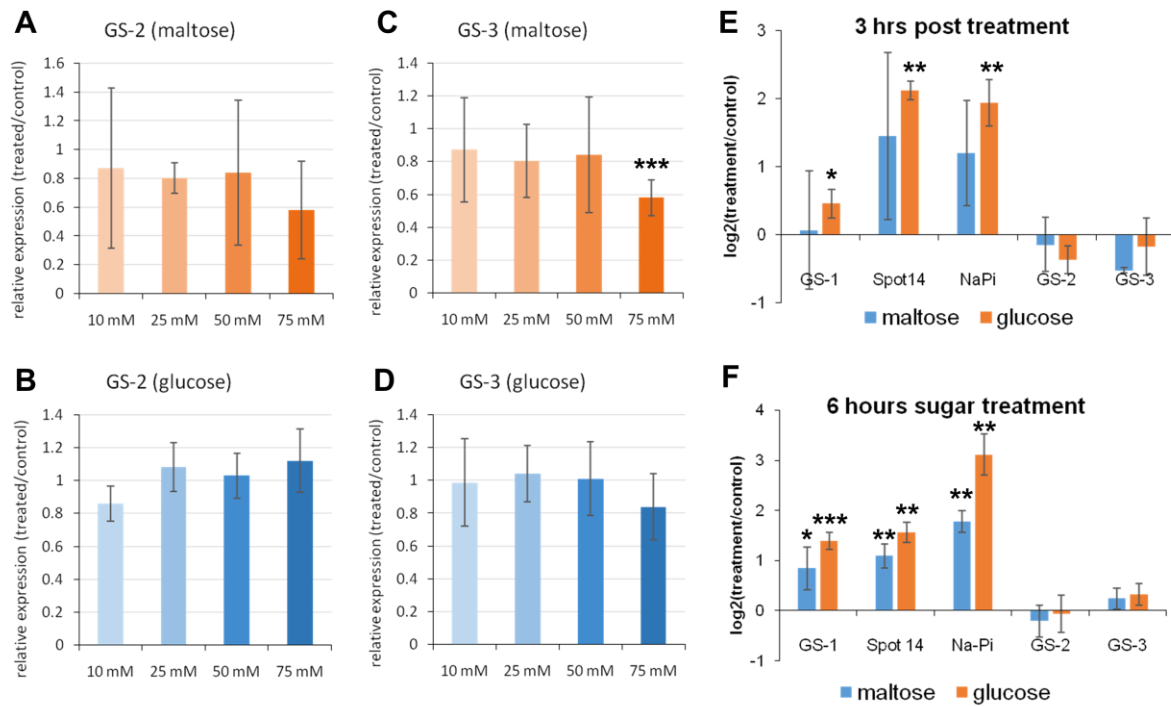
**Figure 2.1.2: Effect of different sugars on the symbiosis specific metabolic genes**

The aposymbiotic animals were treated with 0, 10, 25, 50, and 75 mM of maltose (A)/ glucose (B)/ Sucrose (C)/ galactose (D) for 48 hours prior to RNA isolation and further analysis by qRT-PCR of the target genes. The graphs show the log-ratio of treatment to control. For each treatment, there were three biological replicates, and the results were confirmed by 2 technical replicates. Unpaired T-test (\*p<0.05) and Kruskal-Wallis test (†p<0.05)

### 2.1.5 Conclusion and discussion

The metabolic genes were induced in aposymbiotic animals on 48 hours of treatment with glucose and maltose but not sucrose and galactose, indicating that there is a specificity to maltose and its breakdown product. However, it is possible that the host cannot digest sucrose to show its effect. In the case of galactose, there was a negative effect where rising concentration resulted in the reduction of gene expression. Galactosidase enzyme, responsible for digestion of galactose for energy

production, is known to show substrate inhibition with increasing substrate concentrations (Liao et al., 2016). This further indicates that the gene induction we observe is a metabolic effect, where availability of enough carbon for energy production will shift the ammonium production towards ammonium assimilation and hence the production of glutamine by glutamine synthetase activity.



**Figure 2.1.3: Specificity and rapidity of the response to sugar treatment**

(A-D) The aposymbiotic animals were treated with 0, 10, 25, 50, and 75 mM of glucose/maltose for 48 hours prior to RNA isolation and further analysis by qRT-PCR of all the host's glutamine synthetase genes. Glucose and maltose did not show any effect on the non-symbiosis glutamine synthetase genes (GS-2 and GS-3) with the exception of 75 mM maltose on GS-2. (E,F) The aposymbiotic animals were treated with 75 mM maltose/glucose for 3 or 6 hours prior to RNA isolation. The qRT-PCR results show that some or all of the symbiosis metabolic genes could be induced after 3 or 6 hours with no effect on non-symbiosis glutamine synthetases. Data are presented as the mean  $\pm$  SEM of three biological replicates with two technical replicates. Statistical significance between treatments was determined by unpaired Student's t-test with \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

Out of the three glutamine synthetases, only one (GS-1) was associated with the presence of the native symbiotic *Chlorella* A99. Furthermore, maltose and glucose treatment also showed that only GS-1 is affected while there is no effect on the other two glutamine synthetases. This indicates a differential regulation of the

metabolic genes, where GS-2/3 might be involved in maintaining the cell homeostasis and not in maintaining symbiosis. However, this differential regulation seems to be associated with the ratio of carbon to nitrogen available in the cell as I could show that a simple supplement of sugars can induce the symbiosis genes.

There were two striking observations in these experiments,

- (i) The highest concentration of 100 mM galactose did not affect the host phenotype (**Figure 2.1.1**). This rule out the possibility that other sugars have a deteriorating effect because of hyper-osmosis. However, the exact reason cannot be pinpointed; there is a possibility that dysbiosis is responsible for the diseased phenotype of the aposymbiotic hosts (Lachnit, Bosch, & Deines, 2019). Since higher concentrations of galactose are inhibitory for its own utilization (Liao et al., 2016), it raises a possibility that even the bacterial symbionts are unable to utilize higher concentrations of galactose. While in the case of other sugars, the bacteria are able to utilize them and promote their growth, altering the homeostatic microbial composition and generating a dysbiotic state at very high concentrations of sugars.
- (ii) The induction of the symbiosis genes was strikingly observed even after 3 hours of sugar treatment. It was further interesting to see the trend that glucose had a higher impact as compared to maltose, indicating that the breakdown of maltose precedes the transcriptional induction, shown in the model (**Figure 1.1B**).

## 2.2 Chapter 2: Understanding tripartite symbiosis in *Hydra viridissima*

### Introduction

For over 50 years *Hydra viridissima* has been a subject of research for symbiosis between the host polyp and the endosymbiotic *Chlorella* algae (McAuley, 1986; McAuley & Darrah, 1990; L. Muscatine & Lenhoff, 1963; L. Muscatine, Cook, Pardy, & Pool, 1975; Leonard Muscatine, 1965; Leonard Muscatine & Lenhoff, 1965; Thorington & Margulis, 1981). The mutual exchange of metabolites is the key to the success of this symbiotic relationship. The algae provide fixed carbon in the form of maltose to the host (Cernichiari et al., 1969) that provides a significant competitive advantage compared to the aposymbiotic animals during periods of starvation (Habetha et al., 2003; Huss et al., 1994; L. Muscatine & Lenhoff, 1963; Leonard Muscatine & Lenhoff, 1965; Roffman & Lenhoff, 1969) and promoting oogenesis (Habetha et al., 2003). Moreover, the symbiotic algae have also been shown to protect the host under the temperature stress conditions (Ye, Badhiwala, et al., 2019; Ye, Bhattacharjee, & Siemann, 2019). A hallmark of this strong interdependence is that the algae have lost the autonomy and have to depend on the host for survival; any attempt to cultivate these symbiotic algae has failed (Hamada et al., 2018). Genomic analysis of *Chlorella* A99 revealed the degeneracy in the nitrate assimilation pathway that renders it to depend on the host-derived glutamine as the source of nitrogen (Hamada et al., 2018).

Apart from the symbiotic algae, like other *Hydra* species, the green *Hydra* also harbors a distinct bacterial community. The bacterial composition for any given *Hydra* species is specific and mirrors the phylogenetic relationship of their hosts (Franzenburg, Walter, et al., 2013; Fraune & Bosch, 2007), a pattern termed phyllosymbiosis (Brucker & Bordenstein, 2013). Species-specific antimicrobial peptides of the neuronal origin or the ones regulated by stem cell transcription factor FoxO are involved in shaping *Hydra's* microbiota, which is remarkably stable over time (Franzenburg, Walter, et al., 2013; Fraune & Bosch, 2007; Mortzfeld et al., 2018). Known from other *Hydra* species, the bacteria protect host against the fungal

pathogen infection and even has an effect on the host behavior (Fraune et al., 2015; Murillo-Rincon et al., 2017).

However, the understanding of tripartite interaction comprising of host, algae, and bacteria remains elusive. There have been several studies aiming to elucidate these interactions in other symbiotic systems like corals and symbiotic sea-anemone (Badhai, Ghosh, & Das, 2016; D. G. Bourne, Morrow, & Webster, 2016; Dunphy, Gouhier, Chu, & Vollmer, 2019; Gong, Jin, Ren, Tan, & Xia, 2020; Lima et al., 2020; Meirelles et al., 2018). It was observed that the microbiome composition of the symbiotic and aposymbiotic *Aiptasia* was different (Röthig et al., 2016). Moreover, through *in silico* modeling approach, several putative interactions between the algae and the bacteria have been proposed (Lima et al., 2020). However, in spite of the deep ecological impact of photosynthetic symbioses, such as in the coral-reef system, due to lack of experimental accessibility, the exact phenotypic contribution of these partners in maintaining metaorganism homeostasis is lacking.

In the first part of this chapter, I could demonstrate the role of algal symbionts in selecting the bacterial symbionts and maintain them in a light-dependent manner. Moreover, I could characterize the impact of a shared environment and horizontal transfer of bacteria within a defined metacommunity on the host fitness. Next, I could show that a temporary reduction in fitness ultimately provides community-immunity against invasive bacterial species.

The second part of the chapter focuses on addressing the effect of the host-associated bacterial microbiota on the fitness of the host and the algae and the stability of symbiosis between them. I could demonstrate that there was no direct effect of the bacterial symbionts on the fitness of the algae. Moreover, in some cases, harboring bacteria was costly for the host. Finally, replacement of the normal microbiota with cross-species microbiota had a detrimental effect on the fitness of symbiotic as well as aposymbiotic hosts portraying the importance of harboring native microbiota. Thus, this raises the possibility that in the presence of a stronger and co-evolved core symbiont (photosynthetic algae), the effect of other symbionts (bacteria) can be masked and at instances, they might not have any direct effect at all.

## 2.2.1 Chapter 2.1: Effect of algal symbionts on microbiome composition

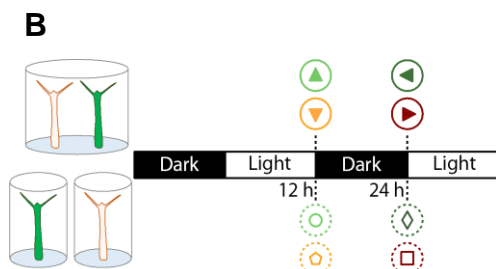
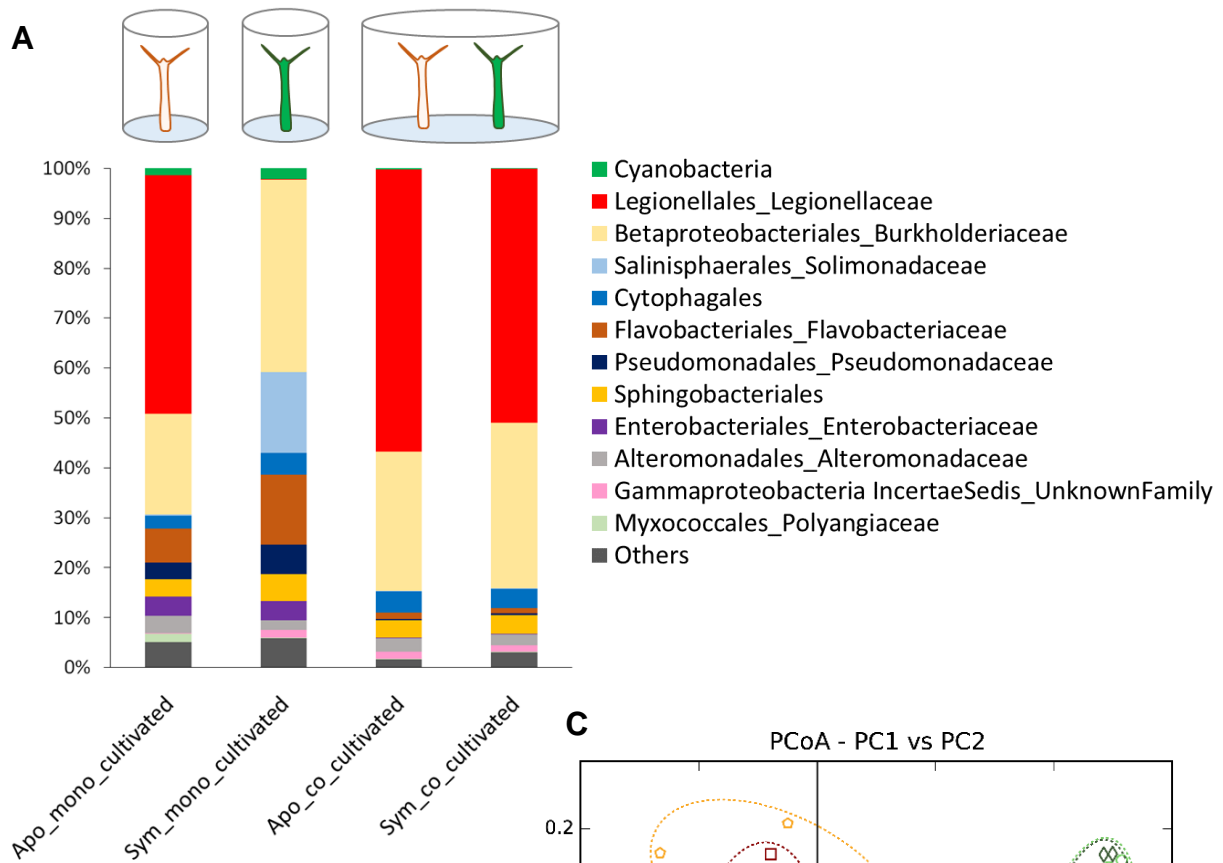
### 2.2.1.1 Objective

The objective of this chapter was to demonstrate the effect of algal symbionts and their photosynthetic activity on the microbiome composition. As observed earlier, the algal symbionts have a profound effect on host physiology. Specifically, it was reported that the secretory products of the algae could also affect the expression of genes in the ectoderm (Hamada et al., 2018). Therefore, I hypothesize that these secretions are likely also to affect the ectodermally associated glycocalyx. Since the glycocalyx inhabits the bacterial symbionts, the secretory products from the host under the influence of the algae or the algal products themselves can have an effect on the bacterial symbionts and hence their composition. The conceivable factors that may play a role here are oxygen levels, sugar/nitrogen-compounds, ROS levels, or the composition of the glycocalyx itself. Collectively, these factors can be impacted by the photosynthetic activity of the algae. Therefore I reasoned that it was obvious to compare the microbiome composition of the symbiotic and aposymbiotic animals to gain compositional insights into the influence of algae on the bacterial composition.

### 2.2.1.2 Results

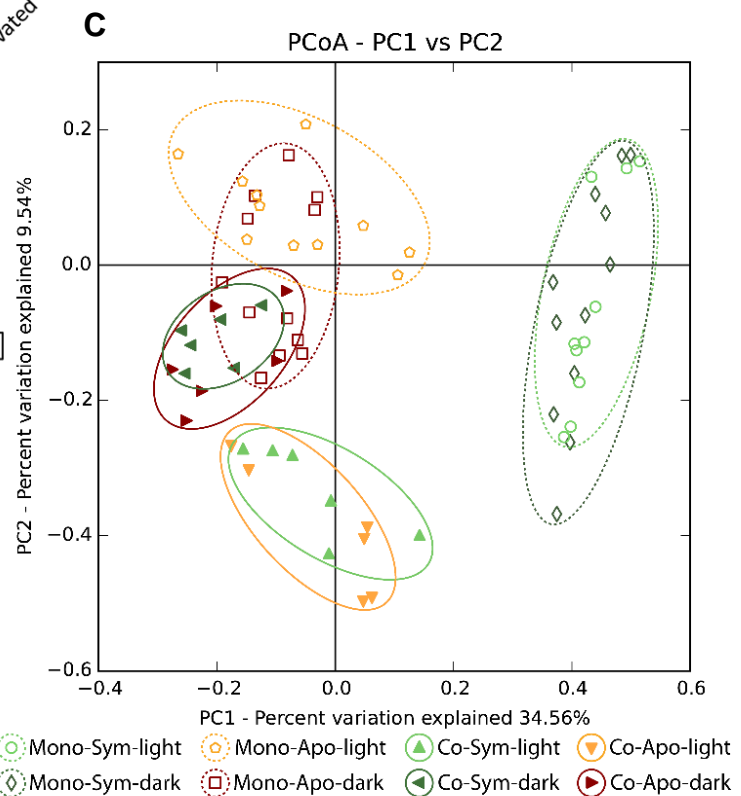
#### **The microbiome composition of symbiotic and aposymbiotic animals is affected by co-cultivation**

Like all *Hydra* species, *H. viridissima* harbors a unique microbiome. 16S rDNA sequencing revealed the different microbiome compositions in the symbiotic and aposymbiotic animals (**Figure 2.2.1.1A**). While *Legionella* sp. occurred in a very low relative abundance (0.03%) in the symbiotic animals, it dominated the aposymbiotic



**Figure 2.2.1.1: Effect of co-cultivation and algal photobiont on the microbiome composition of *H. viridissima* holobiont**

(A) Co-cultivation of symbiotic with aposymbiotic animals drastically affects the microbiome of symbiotic animals, in particular, due to the horizontal transfer of *Legionella* sp. (B) Experimental setup to study the effect of algal photosynthesis on microbiome composition (C) Principal Co-ordinate analysis (PCA) plot showing the Bray-Curtis beta-diversity of microbiome composition. Transfer of bacteria from aposymbiotic to symbiotic animals in the absence of algal photosynthesis (in dark) makes symbiotic polyps more similar to aposymbiotic polyps. During the day, in the presence of algal photosynthesis, the microbiome of the symbiotic animals shifts towards the core symbiotic microbiome.



microbiome up to 47.8%. Both the lines maintained their distinct microbiome signatures when cultivated separately. However, upon co-cultivation (**Figure 2.2.1.1A**), I observed an increased abundance of *Legionella sp.* in the symbiotic animals, which might be the result of horizontal transfer of bacteria from aposymbiotic to symbiotic animals. This *Legionella sp.* OTU dominated up to 50.9% in co-cultivated symbiotic animals. This shows the invasive nature of *Legionella sp.*

### **The microbiome composition differs between light and darkness**

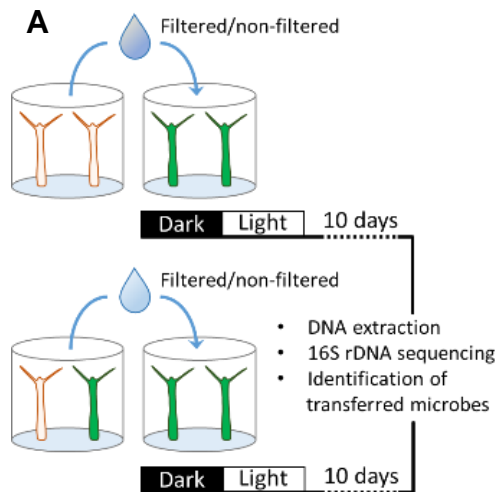
The preliminary experiments performed by former lab members- Dr. Sebastian Fraune and Dr. Katja Schroeder, revealed the effect of algal photosynthesis on the altered microbiome of the symbiotic animals (Schroeder et al., unpublished). They performed the experiment under co-cultivation and observed light dependant shifts in the microbiome composition (**Figure 2.2.1.1B/C**). By integrating our results, displayed on the same PCA plot in **Figure 2.2.1.1C**, we could show that under the influence of algal photosynthesis, during day time, the microbiome of the symbiotic animals under co-cultivation shifted towards the core microbiome of mono-cultivated symbiotic animals. A similar trend was observed in the case of co-cultivated aposymbiotic animals because after prolonged co-cultivation (4 weeks), they gained algal symbionts from the symbiotic animals. On the other hand, the mono-cultivated animals maintained a stable microbiome irrespective of the light conditions.

### ***Legionella* can be transferred through water**

The transmittability of the microbes in a shared environment has been documented for several animal systems, including aquatic systems, as exemplified in zebrafish (Burns et al., 2017). I tested if I can transfer the bacteria by supplementing only the water of aposymbiotic animals to the mono-cultivated symbiotic animals. As shown in the **Figure 2.2.1.2A**, the symbiotic animals were cultivated in the filtered/non-filtered water from aposymbiotic or long-term (8 weeks) co-cultivated animals. It was observed that the *Legionella sp.* could be transferred through the non-filtered water from the aposymbiotic animals (**Figure 2.2.1.2B**), indicating the role of dispersal of bacteria in a shared environment. It was noteworthy that, even

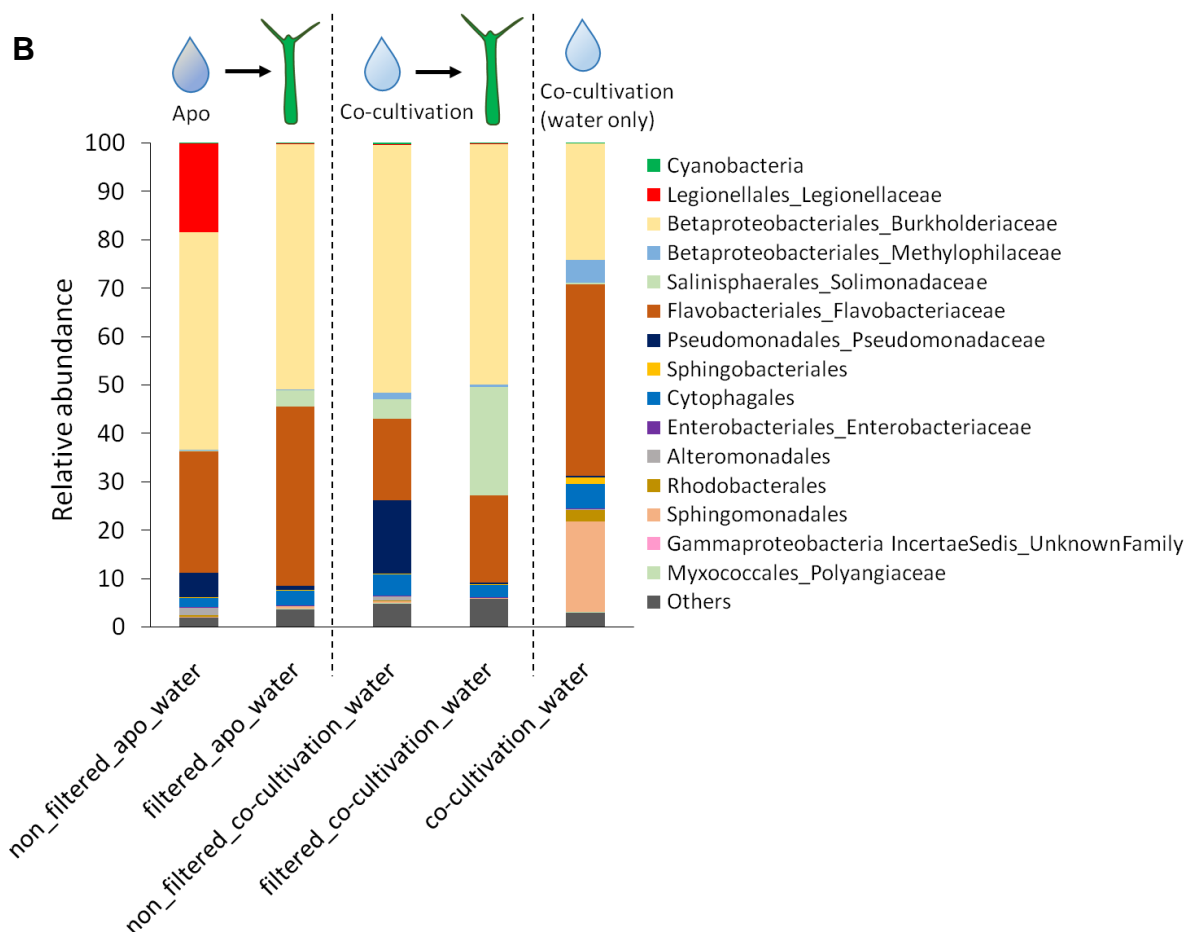


though the co-cultivated animals were colonized with the *Legionella sp.*, it could not be transferred through their water, and it was absent in the water itself (**Figure 2.2.1.2B**). This indicated the absence of dispersal of bacteria under long-term co-cultivation.



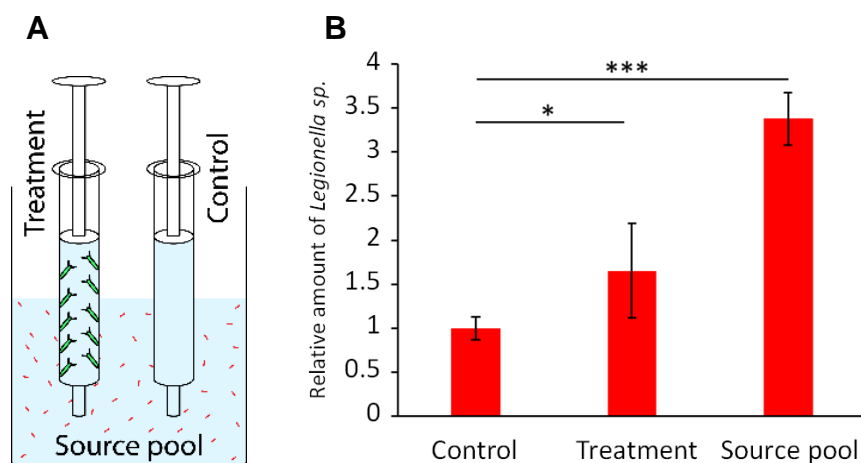
**Figure 2.2.1.2: *Legionella sp.* is transferrable through water**

(A) Experimental setup to study the horizontal transfer of *Legionella sp.* The mono-housed symbiotic animals were incubated in water from the aposymbiotic and co-housed animals for ten days in the 12h-12h dark-light cycle. The water filtered with 0.2  $\mu$  filter was used as control. (B) Taxa summary plots showing microbiome composition of the symbiotic animals treated with filtered/non-filtered water from aposymbiotic animals and co-housed animals along with microbiome composition of the co-housing water. Symbiotic animals treated with non-filtered aposymbiotic water acquired *Legionella sp.* There was no *Legionella sp.* acquisition from co-housing water, and it was absent in the water itself.



### ***Legionella sp.* migrates actively towards the symbiotic animals**

Several *Legionella* species have been shown to be flagellated and have the genetic potential to show a chemotactic response (Appelt & Heuner, 2017). In order to study if this symbiotic *Legionella sp.* can migrate towards the symbiotic animals actively, I performed a migration assay (Dennis, Seymour, Kumbun, & Tyson, 2013) (**Figure 2.2.1.3A/B**). Therefore, 1ml syringes were filled with sterile water and placed in the “source pool” that contained the water pre-incubated with aposymbiotic animals. As a potential target, the treatment syringes also contained ten symbiotic polyps each (**Figure 2.2.1.3A**). Upon 48 hours, I quantified the amount of *Legionella* migrated into the syringe water using quantitative PCR. This revealed a significantly increased ( $p$ -value<0.001) migration of *Legionella sp.* in the treatment syringe containing the symbiotic animals as compared to the control syringe (**Figure 2.2.1.3B**). Together, this indicated active chemotaxis of *Legionella sp.* towards the symbiotic animals.



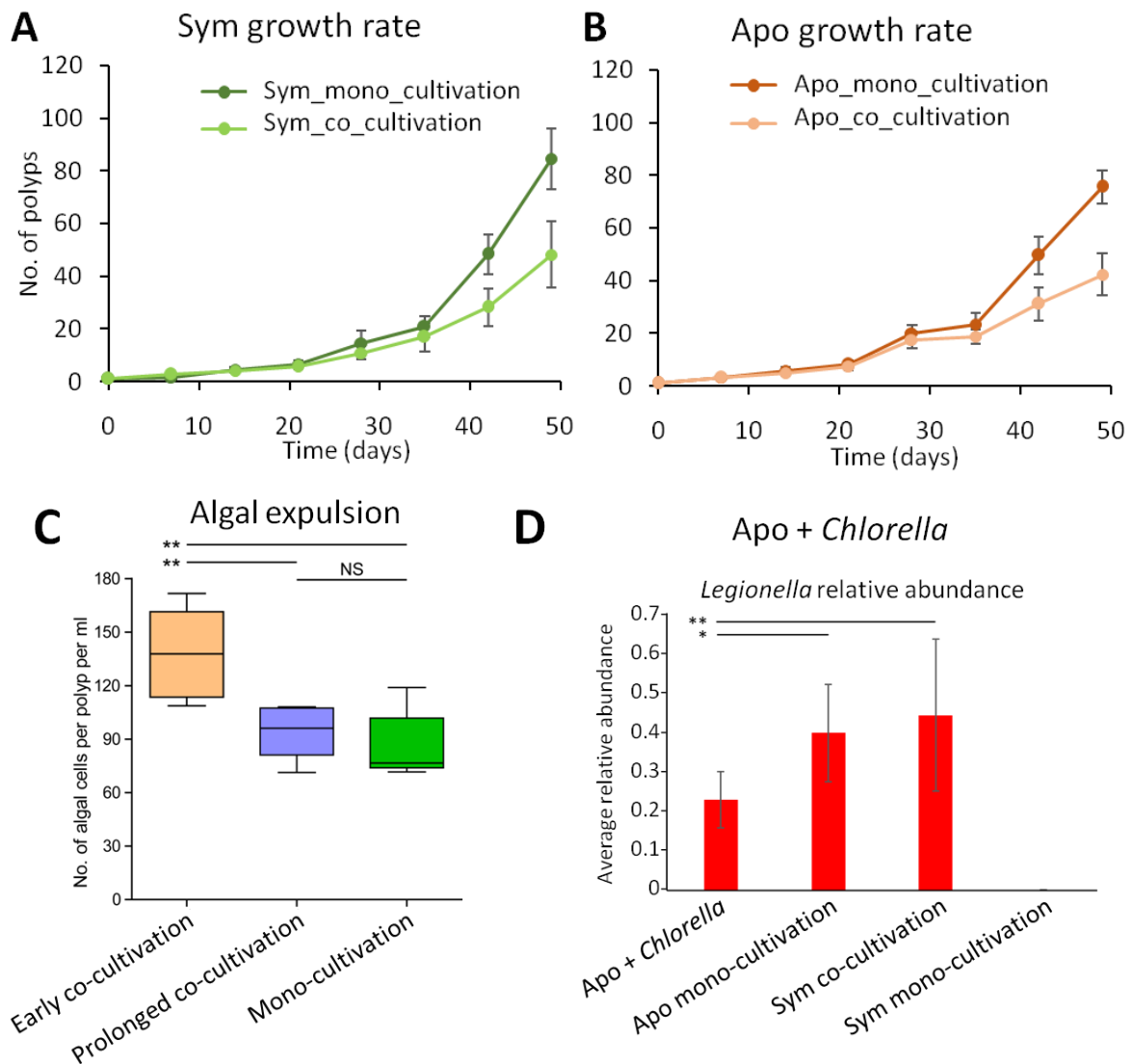
**Figure 2.2.1.3: *Legionella sp.* displays targeted migration towards the symbiotic host**

(A) Experimental setup for the migration assay. The treatment syringe held symbiotic polyps, and the control syringe was without polyps. The source pool is the water from aposymbiotic animals. (B) qPCR with *Legionella sp. Hvir* specific primers showed an increased migration in the treatment syringe as compared to the control ( $n=6$ , t-test,  $*p<0.05$ ,  $***p<0.001$ ).

### **Transfer of microbes negatively influences the host-fitness**

An altered microbiome composition or a dysbiotic state has a negative fitness cost to the host (Tiffany & Bäumler, 2019). I assayed if the transferred microbes have any fitness cost for the host by comparing the population growth rates. The acquisition of *Legionella sp.* reduced the population growth rates in the co-cultivated

symbiotic animals (**Figure 2.2.1.4A**). It is apparent from the data that the establishment of new algal symbiosis in aposymbiotic animals is an energetically expensive process. Hence, it is reflected in the reduced growth rate of the co-cultivated aposymbiotic animals as well (**Figure 2.2.1.4B**). Therefore, the acquisition of new bacterial symbionts by the symbiotic animals seems to be stressful for the holobiont.



**Figure 2.2.1.4: Acquired microbes affect host fitness**

(A) Colonization of symbiotic animals by *Legionella sp.* under co-cultivation is fitness costly for the host resulting in a reduced growth rate. (B) The co-housed aposymbiotic animals also had a reduced growth rate that can be accounted for by the establishment of new symbiosis with the expelled algae from the symbiotic host. (A,B Error bars indicate standard deviation, n=5) (C) Immediate early acquisition of *Legionella sp.* results in an increased expulsion of algae as compared to prolonged co-housing and mono-housing of symbiotic animals. (n= 5, t-test, \*\*p<0.01). (F) Acquisition of *Chlorella* negatively influences *Legionella sp.* abundance in aposymbiotic animals (n=6, Student's t-test, \*p<0.05, \*\*p<0.01)

### **Increase in algal expulsion during early co-cultivation**

The symbiotic animals have a tendency to actively release the algal symbionts in the surrounding water to control the intracellular algal population (Baghdasarian & Muscatine, 2000; L Muscatine & Pool, 1979). A similar phenomenon can be observed in response to stress where algal symbionts leave the host cell (McAuley, 1981), also observed in coral bleaching. Since *Legionella sp.* is fitness costly for the host (**Figure 2.2.1.4A**), I investigated its effect on the algal expulsion by incubating an equal number of polyps in an equal amount of sterile water for 24 hours (see **Materials and Methods**). Under co-cultivation, the symbiotic animals in the early phase of *Legionella sp.* acquisition (4 days post co-cultivation), had higher levels of algal expulsion per polyp as compared to mono-cultivated and long-term co-cultivated (8 weeks) animals (**Figure 2.2.1.4C**). This indicated that the phase of *Legionella sp.* acquisition has fitness cost for the host that might initiate the stress response and releases the algal symbionts in the surrounding.

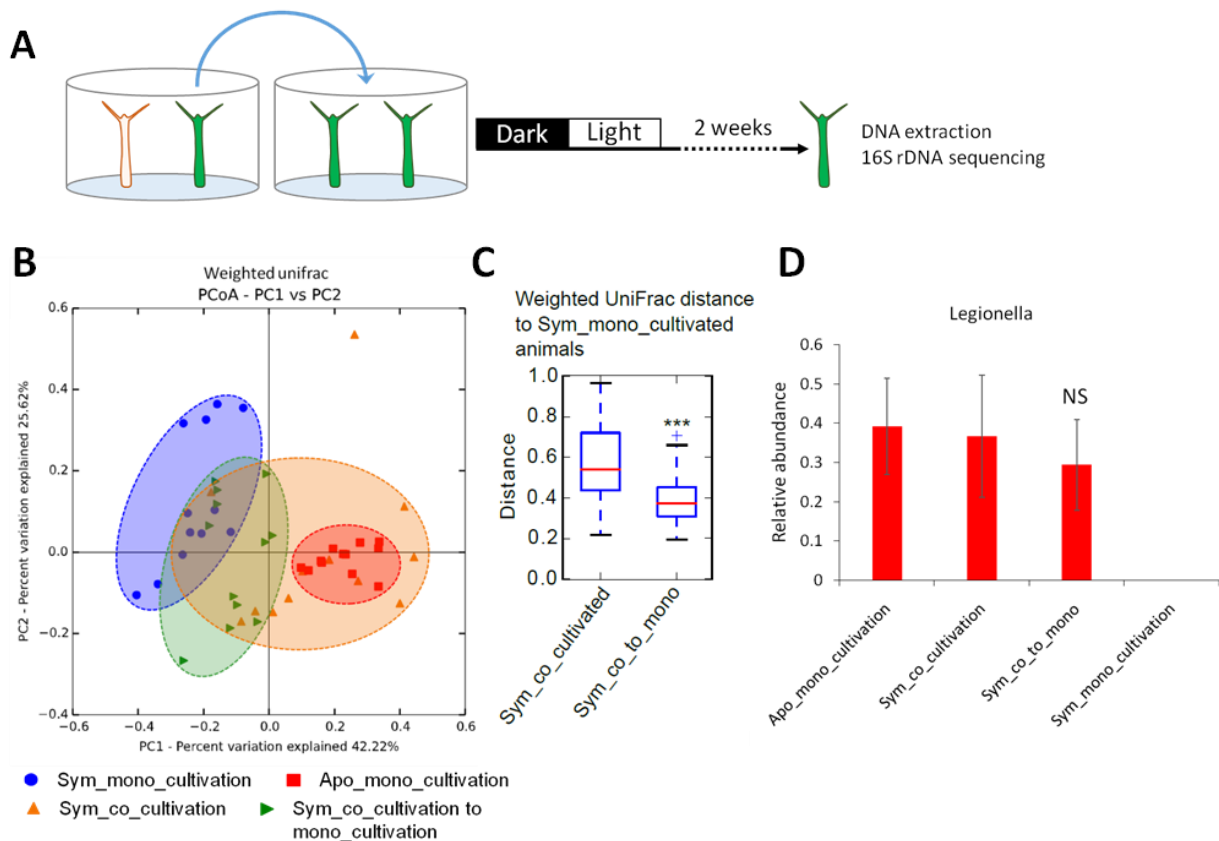
### **Acquisition of *Chlorella* negatively affects *Legionella sp.* abundance**

The expelled algae by the symbiotic animals can also act as a means of horizontal transfer of the symbionts (Miyokawa et al., 2018). These expelled cells can be taken up by the aposymbiotic animals and be converted to symbiotic animals. In order to study the effect of these acquired symbiotic algae on the *Legionella sp.*, I force-fed the aposymbiotic animals with the freshly isolated symbiotic algae and observed the microbial composition of the newly formed symbiotic animals. After eight weeks, the relative abundance of *Legionella sp.* reduced in the aposymbiotic animals fed with algae as compared to the controls (**Figure 2.2.1.4D**).

### **Shifting the co-cultivated animals to mono-cultivation restores the microbiome**

If the shift observed in the co-cultivated symbiotic animals is only subjective to co-cultivation with the aposymbiotic animals, the removal from co-cultivation should result in the restoration of the microbial composition. The long-term (8weeks) co-cultivated symbiotic animals were transferred to mono-cultivation (**Figure 2.2.1.5A**) for two weeks, and the microbiome composition was compared to the mono- and co-cultivated animals. As shown in **Figure 2.2.1.5B/C**, the weighted unifracs distance

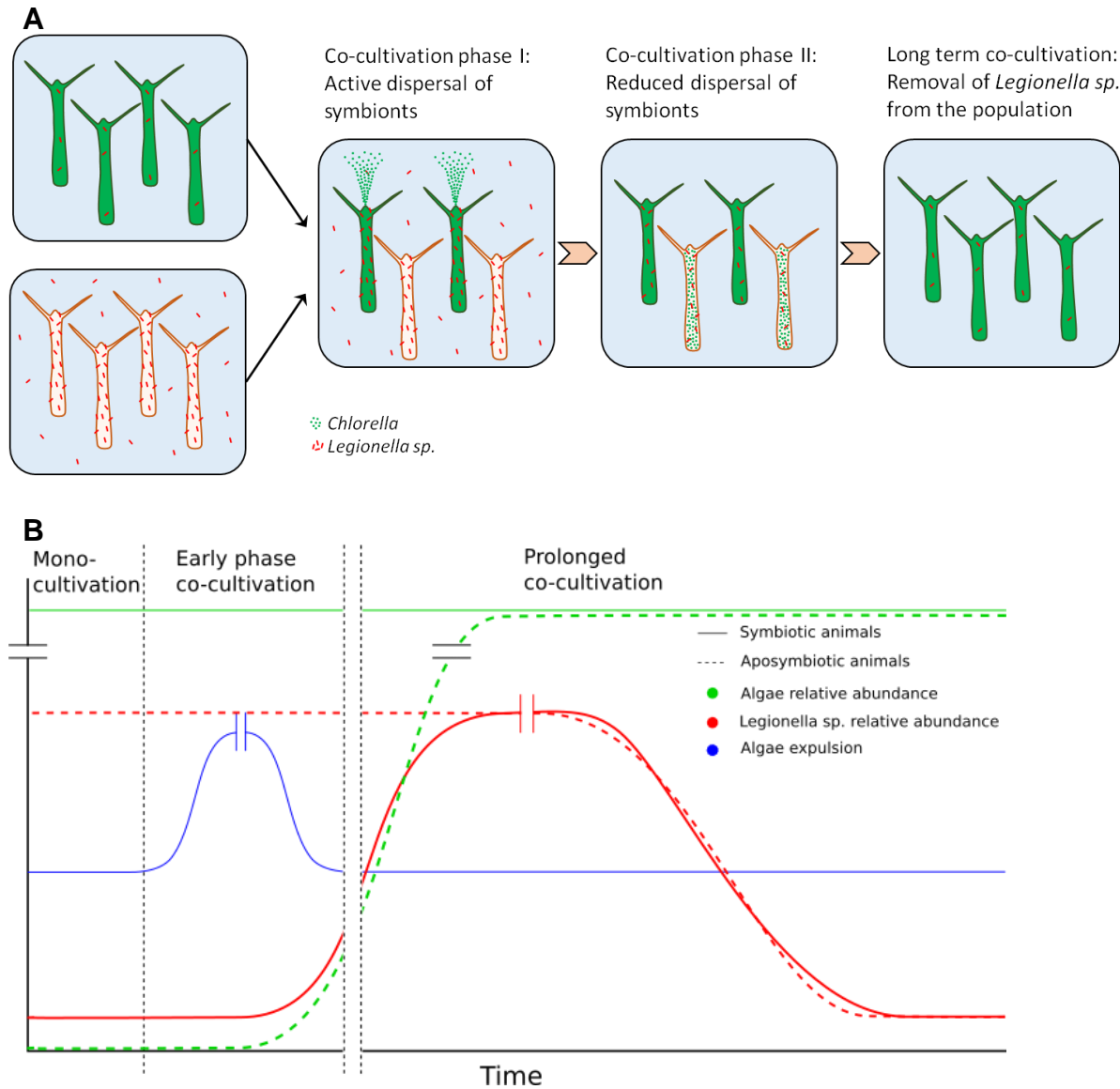
between the mono-cultivated animals and the animals removed from the co-cultivation, significantly reduced ( $p$ -value $<0.001$  as compared to the co-cultivated symbiotic animals. This indicated the restoration of the core symbiotic microbiome. It was noteworthy, after two weeks of removal from co-cultivation, while there was a small trend in the reduction of *Legionella* average relative abundance (**Figure 2.2.1.5D**), it was statistically insignificant. A longer incubation might have resulted in a further reduction in the average relative abundance.



**Figure 2.2.1.5: Effect of transferring the co-cultivated animals to mono-cultivation**

(A) The co-cultivated symbiotic animals were transferred to mono-cultivation for two weeks. (B) The PCA plot showing a weighted UniFrac matrix. (C) The distance of the symbiotic animals transferred to mono-cultivation (Sym\_co\_to\_mono) to the mono-cultivated symbiotic animals was significantly lesser than the co-cultivated symbiotic animals (t-test,  $p$ -value Bonferroni correction,  $***p<0.001$ ). (D) There was no reduction observed after just two weeks of removal from the co-cultivation.

Thus, as summarized in **Figure 2.2.1.6 A/B**, I could show that the acquisition of the invasive bacterial symbionts acts as a signal to disperse the algal symbionts to provide community-immunity to the *H. viridissima* population.



**Figure 2.2.1.6: Summary models explaining the role of horizontal transfer of symbionts and control of invasive species in the metacommunity.**

(A,B) The mono-cultivated animals maintain their respective levels of symbionts. Upon co-cultivation, in the early phase (phase-I), there is an increased algal expulsion upon *Legionella* acquisition by symbiotic animals. This alga is taken up by the aposymbiotic animals, and under phase II, there is a reduction in the dispersal of the symbionts. After prolonged co-housing, the algal abundance in aposymbiotic animals would increase to a level of symbiotic animals, and the levels of *Legionella* will plummet. (B) represents the graphical summary with arbitrary units.

### 2.2.1.3 Discussion

In recent years, biologists have developed a growing appreciation for complex host-symbiont interactions. Symbiosis research traditionally focused on bipartite host-microbe interactions such as the squid-*Vibrio fischeri* symbiosis, while tripartite and multipartite associations have received less attention. However, it is becoming evident that long-term symbiotic persistence is prevalent not only in two-party interactions but also in more complex systems such as reef-building corals. Corals represent a complex multipartite interkingdom symbiosis involving the coral polyp, eukaryotic endosymbiotic algae of the genus *Symbiodinium*, and a vast array of intracellular and extracellular bacterial colonizers, viruses, fungi and apicomplexans (Lema, Willis, & Bourne, 2012; Rohwer, Seguritan, Azam, & Knowlton, 2002). The documented decline in coral reef ecosystems worldwide (Carpenter et al., 2008) gives researchers a new urgency to understand how stability and breakdown ('coral bleaching') of these multipartite symbioses are influenced by changing environmental conditions. When investigating coral bleaching, most researchers have initially focused on the loss of the algal symbionts, which correlates with declining coral health. However, with the discovery that diseased or bleached corals harbor different bacterial communities than healthy ones (D. Bourne, Iida, Uthicke, & Smith-Keune, 2008; Perez, 2006; Reis et al., 2009; Ritchie, 2006; Sunagawa et al., 2009), the coral holobiont was recognized as a tripartite association. Since then bacterial diversity in corals has been extensively studied and putative functions of the coral microbiota have been proposed including pathogen defense (D. G. Bourne et al., 2016; Lima et al., 2020; Rohwer et al., 2002; Shnit-Orland & Kushmaro, 2009) and the supply and cycling of nutrients (Lesser, Mazel, Gorbunov, & Falkowski, 2004; Räddecker, Pogoreutz, Voolstra, Wiedenmann, & Wild, 2015; Wegley, Edwards, Rodriguez-Brito, Liu, & Rohwer, 2007). Nevertheless, compared to the well-studied coral-algal symbiosis, the current understandings of the coral-bacterial partnership are still limited and even less for algal-bacterial interactions. However, it has become clear that corals need to be studied as holobionts to fully assess the contribution of each partner to coral bleaching and elucidate their role in adaptation to environmental stress.

Given the toolkit available for studying symbiotic interactions in the model organism *Hydra* (Augustin, Fraune, Franzenburg, & Bosch, 2012; Bosch, 2012; Hamada et al., 2018), the green hydra, *H. viridissima*, offers a promising system for deciphering the underlying mechanisms in tripartite associations comprising a cnidarian host, endosymbiotic photosynthetic algae and a stably associated bacterial microbiota.

The present study has revealed a unique aspect of this tripartite interkingdom symbiosis. We could show for the first time, to our knowledge, the role of the photosynthetic symbiont, in combination with inter-host dispersal, in shaping the host bacterial community in a light-dependent manner. Yet, we lack any mechanistic understanding of the interaction between the endodermally located intracellular algae and the extracellularly residing bacteria that inhabit the mucus-like layer of the ectodermal glycocalyx (Fraune et al., 2015). Although the *Chlorella* photobiont and the bacterial microbiota colonize separate niches in the *H. viridissima* holobiont, it seems likely that the photosynthetic activity of the endosymbiotic algae can influence microenvironmental physicochemical parameters such as sugar, pH and oxygen level. Therefore, the photosynthetic endosymbiont may modify the properties of microbial habitat, which is supported by studies in corals indicating that the mucus of some corals cycles from being fully-oxygenated during photosynthesis of the coral endosymbiont *Symbiodinium* to anaerobic at night when respiration rates exceed oxygen production (Brown & Bythell, 2005; Carlton & Richardson, 1995; Kuhl, Cohen, Dalsgaard, Jorgensen, & Revsbech, 1995). Furthermore, *Symbiodinium* endosymbionts were shown to influence the microbial community structure by the release of complex carbon-containing secretions, including dimethylsulfoniopropionate (J.-B. Raina, Dinsdale, Willis, & Bourne, 2010; J. B. Raina, Tapiolas, Willis, & Bourne, 2009). Besides being directly affected by photosynthesis products, the microbial niche may also be modified due to photosynthesis-induced changes in host metabolism. Indeed, photosynthetic activity of *Chlorella* has been shown to involve transcriptional changes not only in the endoderm of *H. viridissima* but also in the ectoderm that gives rise to the microbial habitat, the glycocalyx (Hamada et al., 2018).



Moreover, I could show the effect of shared environment and horizontal transmission of bacteria resulting in a dramatic alteration of the microbiome in symbiotic animals. Under the impact of algal photosynthesis, we observed the diurnal changes in the microbiome composition. Interestingly the diurnal fluctuations became apparent only when the microbiome of the symbiotic animals changed, i.e., under co-cultivation. Upon horizontal transfer of bacteria, the microbiome composition of the symbiotic animals resembles the aposymbiotic microbiome. However, at this point, the algal photosynthesis tends to restore the microbiome to its native state of mono-cultivated symbiotic animals in a light-dependant manner. Taken together, our results indicate that the photosynthetic activity of the *Chlorella* endosymbiont is involved in shaping the bacterial microbiota of *H. viridissima*. However, currently, it is unknown whether the light-dependent microbial shift in green *Hydra* plays a physiological role and has functional consequences for the holobiont phenotype. A similar observation has been made in case of coral where the bacterial composition alters drastically between day and night (Kelly et al., 2019). Since there were no corresponding changes in the surrounding water, the observed effects were the result of algal photosynthesis or host intrinsic properties.

Moreover, the chemotaxis experiment showed that *Legionella sp.* migrates actively towards the symbiotic hosts, which can be in response to the secretory products of the algae. Irrespective of possessing flagella, some *Legionella* species are known to show swarming behavior (Appelt & Heuner, 2017). Moreover, many *Legionella* species are known to possess chemotaxis genes (Appelt & Heuner, 2017), but it has never been shown experimentally to show chemotaxis. Here I report for the first time, to my knowledge, the chemotaxis movement of a host-associated *Legionella sp.* towards the symbiotic *Hydra viridissima* polyp.

Invasion by *Legionella sp.* might activate the host's immune response that can be mediated via LRR/NLR – myd88 signaling (Bosch et al., 2009). Since the bacteria are able to colonize the host, especially the aposymbiotic host, it is less likely that the host exercises the defense response to the full extent. This colonization by *Legionella sp.* proved to be fitness costly for the symbiotic host, resulting in a reduction of the population growth rate. This can be the result of a switch in resource investment from the native symbionts to the incoming invasive symbionts. The

resource investment can be in the form of defense response from the host or an increased nutrient scavenging by the incoming bacteria.

The exact mechanism yet unknown, the invading bacteria, in their early phase of colonization, result in an increased algal expulsion by the symbiotic hosts. The presence of the incoming bacteria might be a stress signal for the host, in response to which it propagates the algal symbionts in the surrounding water to be taken up by the aposymbiotic animals.

Furthermore, uptake of the algal symbionts by the aposymbiotic animals resulted in a reduction of the *Legionella sp.* abundance. It might be the result of a higher metabolic waste produced by the host in the presence of algal symbionts, or the presence of algae might facilitate the growth of other symbiotic bacteria in the longer-term.

Interestingly, the dispersal of the *Legionella sp.* stopped after a prolonged co-housing, the stage at which the aposymbiotic animals were already in the process of being converted to symbiotic state. This represents a population-wide response in response to the acquisition of the algal symbionts. Combined with the chemotaxis results, it is suggestive that the symbiotic animals attract the invasive bacterium, ultimately stopping its dispersal in the population.

Summarized in **Figure 2.2.1.6**, I could show that the *Legionella sp.* is dispersed from the aposymbiotic animals. They migrate towards the symbiotic animals actively, possibly towards the secretory products of the algae. We could show how algae play an important role in modulating the microbial composition of the host. It aids in attracting the invasive *Legionella sp.* ultimately leading to “stop” its dispersal and control the abundance, thereby providing the “community-immunity,”; where the holobiont undergoes a temporary fitness reduction to provide long-term protection to the population.

One can extend the reported phenomenon to a recently published novel hypothesis about the communicability of ‘non-communicable’ diseases (Finlay, 2020). It expands on the fact that in a shared environment, through the horizontal transmission of bacteria, the classically “non-communicable” diseases that are impacted by the altered microbiome can be communicated as well. Here we showed

that co-cultivation impacts the microbiome to a great extent. The horizontal transfer of bacteria in a shared environment resulted in a fitness alteration of the host.

This study also highlights the fact that in order to describe the functionality of a metaorganism, it becomes vital and prudent to take into consideration the microorganisms present in the surrounding environment as well as associated with the other hosts in the shared niche and understand it from a metacommunity perspective (Miller, Svanbäck, & Bohannan, 2018).

## 2.2.2 Chapter 2.2: Effect of bacteria on host-algal symbiosis

### 2.2.2.1 Objective

The objective of this chapter was to address the effect of the bacterial symbionts on the host-algal symbiosis. As mentioned in the introduction, the bacteria have a profound effect on the host physiology from the simplest multicellular organisms to a complex being as humans. It has been observed in *Hydra* to have an effect on host behavior, i.e., spontaneous contractions as well as protection against pathogens. Moreover, it has been observed in *Hydra oligactis* that inter-bacterial interactions are responsible for the alteration of the tissue homeostasis resulting in the formation of tumors. Thus the bacterial symbionts have been shown to have a profound effect in other species of *Hydra*, but not in green *Hydra* that harbors the endosymbiotic algae. The aim was to study how the extracellularly located bacteria on the ectodermal glycocalyx have an effect on the endodermally located endosymbiotic algae. This was primarily tested by comparing the germ-free (bacteria-free) animals with the control animals (DMSO, Conventionalized, and Wild animals). The following fitness parameters were assessed:

- Algal density in symbiotic animals
- *In vivo* photosynthetic yield
- Population growth rate
- Symbiogenesis in aposymbiotic animals
- Response to starvation
- Temperature stress
- Pathogen infection
- Phototaxis in symbiotic animals

However, the results of these comparisons were inconclusive. So, in order to delve further into the causative effect of bacteria, I replaced the normal microbiome with a complete set of bacteria from other *Hydra* species, *H. vulgaris* AEP and *H. oligactis*. The stress response and physiological parameter like growth were assessed to study the effect of the altered microbiome on the host-algal symbiosis.

## 2.2.2.2 Results

### Generation of germ-free animals

To gain in the first insights into the phenotypic effect of the microbiota on the host-algal symbiosis, I generated germ-free (bacteria-free) *H. viridissima* animals by treatment with antibiotics. The animals were treated with 0.1 mg/ml each of ampicillin, streptomycin, neomycin, and rifampicin and 0.12 mg/ml of spectinomycin (Fraune et al., 2015).

Several trials were carried out to generate the germ-free animals. The conventional protocol (Fraune et al., 2015) of continuous antibiotic treatment for 15-17 days resulted in weak and dead animals at the end of the treatment. Those animals could not be utilized for any further experimental work, nor were those animals in any condition to be able to feed.

In another trial, the animals were treated with a higher antibiotics concentration up to 10x with reduced treatment time, but it resulted in either dead animals at very high concentrations (10x) or there were still bacteria persisting at the end of 12 days treatment at lower concentration (2x to 5x).

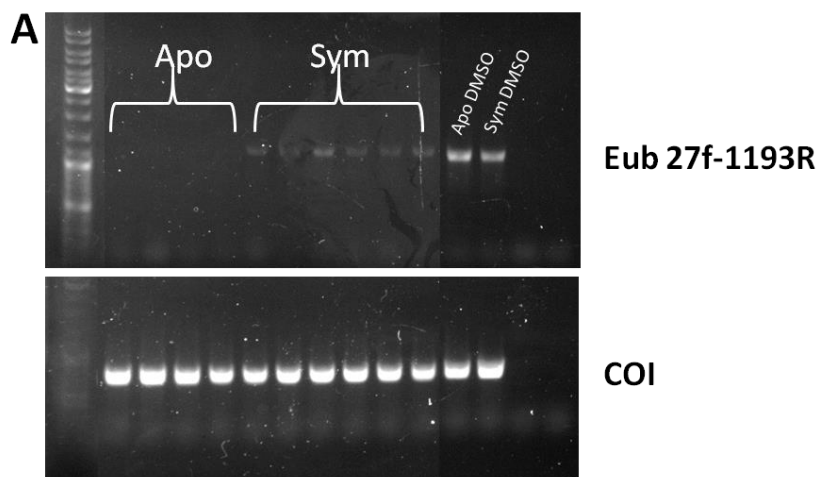
Another trial involved treating the animals with a normal concentration of antibiotics with alternating periods of treatment and feeding. It included three days of treatment followed by feeding with germ-free *Artemia* without antibiotics (He *et al.*, unpublished) and putting them back in antibiotics the next day after cleaning. It looked promising; however, it gave rise to an antibiotic-resistant *Pseudomonas* strain. So those animals had to be discarded.

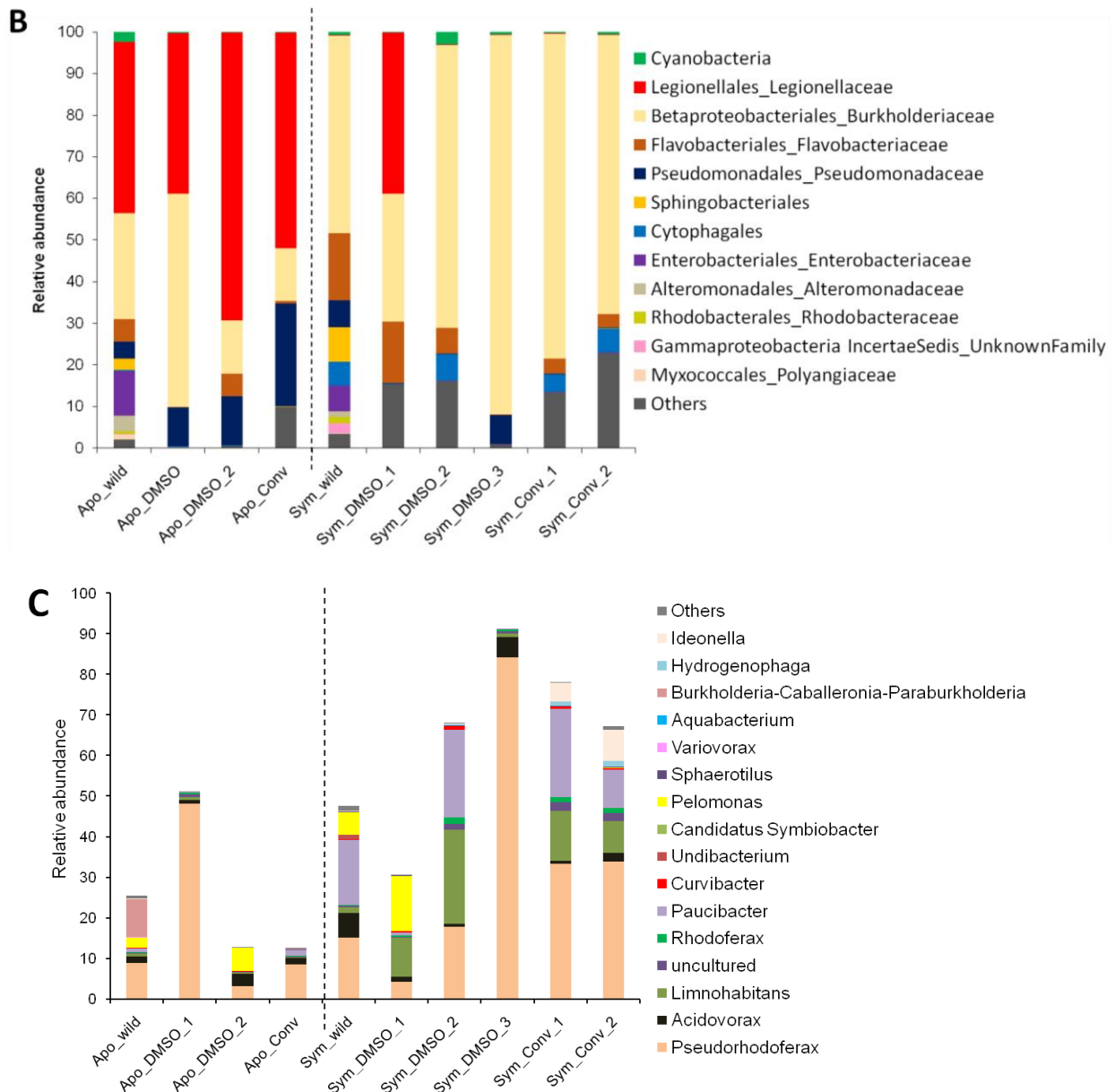
It should be noted that in all the trials as mentioned above, the antibiotic solution of the animals was changed on alternate days, and the animals were removed by forceful pipetting to change the container.

Considering all the attempts, as mentioned earlier and their limitations, the following working protocol was devised. The animals were treated with 1.2x antibiotic solution that was changed daily. The antibiotic solution was carefully decanted without removing the animals, to avoid damaging them. They were fed after one

week of treatment with germ-free *Artemia*. The next day, the container was changed, and the animals were placed in a fresh antibiotic solution and a new container. The process was continued for 6 weeks. Initially, the sterility was assayed by plating a homogenized polyp on R2A agar plate twice a week and by PCR based confirmation of 16S rDNA. Since the classical set of primers to check the bacterial presence used previously (Fraune et al., 2015), 16S 27F-1492R, could amplify the chloroplast 16S, they could not be utilized to confirm the sterility of the symbiotic animals due to the presence of the algal symbionts. So, instead, 16S 27F-1193R primer set (Beckers et al., 2016) was used for the confirmation (**Figure 2.2.2.1A**). However, for the germ-free symbiotic animals, additional confirmation was made by 16S rDNA sequencing, as there was some amplification observable (**Figure 2.2.2.1A**) even when there were no colonies on R2A plates from the polyps. The sequencing results showed maximum 150 bacterial reads, out of average 50,000 total reads for symbiotic animals, which were not part of the symbiotic bacterial community.

As controls, a set of animals was treated with a respective concentration of dimethyl sulfoxide (DMSO) as solvent control. After the generation of the germ-free animals, a subset of these animals was given back the complete set of the bacteria from the wild animals. These animals were termed as conventionalized animals. Both these controls set of animals were maintained in the sterile water and fed with germ-free shrimps on a schedule the same as the germ-free animals. Furthermore, these control animals were checked for their microbiome composition at different time points every two months (points 1, 2, and 3) after maintaining under germ-free feeding regime (**Figure 2.2.2.1B/C**).





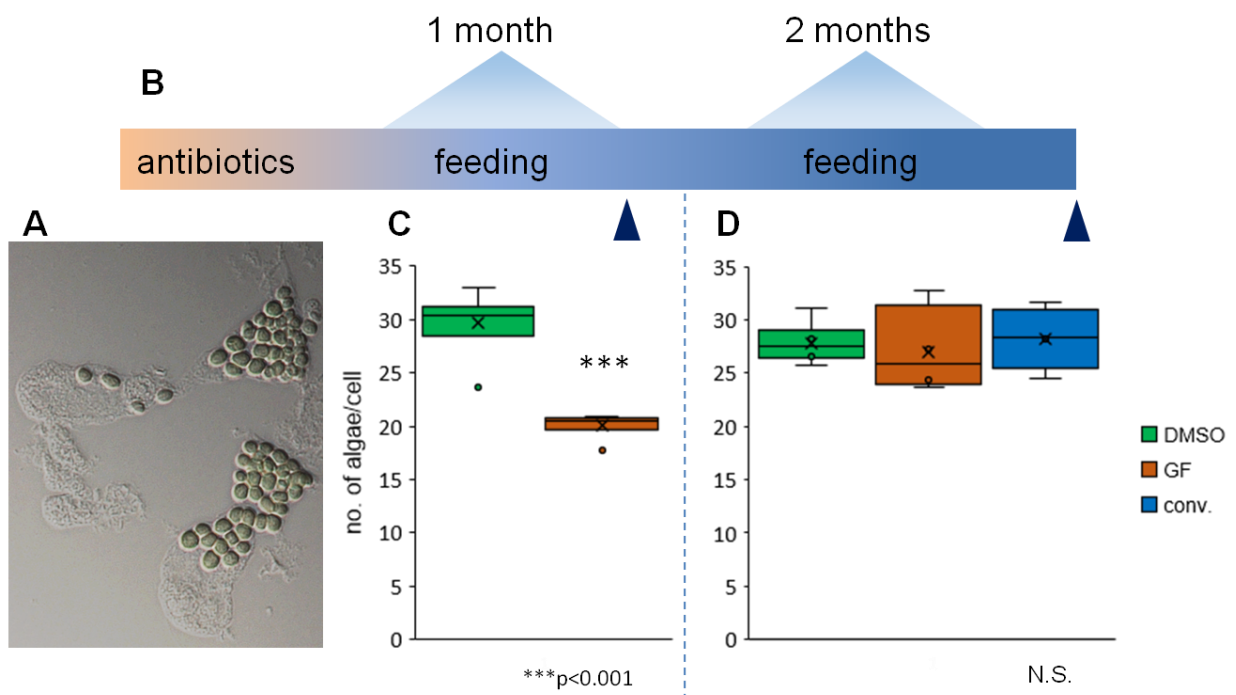
**Figure 2.2.2.1: Assessment of the microbiome composition of the DMSO/Conventionalized control and wild animals**

(A) The gel picture represents PCR for the 16S rDNA gene with primers 27F-1193R to confirm the presence of bacteria on the germ-free hosts. Since symbiotic germ-free animals showed the interference of the algal chloroplast DNA, their germ-freeness was confirmed by 16S rDNA sequencing. (B) The graph represents the relative abundances of the bacteria at the family level for the DMSO and conventionalized animals. For reference, the composition of wild animals is provided. The composition of the *Burkholderiaceae* family at the genus level is shown in (C). Both graphs indicate the variation in the DMSO/Conventionalized animals after long-term maintenance with germ-free feeding.

### Effect of bacteria on the algal density

Algal density, defined here as the average number of algal cells per host endodermal cell, is a credible measure of the host and algal fitness. As observed in case of other *Cnidarians* like corals, any kind of stressors or nutritional imbalance would lead to the bleaching of the algae from the host (Karim et al., 2015; Morris, Voolstra, Quigley, Bourne, & Bay, 2019; Pogoreutz et al., 2017). Similarly, in the case of *Hydra*, I hypothesized that if bacteria have a profound effect on the host-algal symbiosis, their absence would lead to a homeostatic imbalance and loss of algae.

Indeed, it was apparent that the animals treated with antibiotics to have visually lost the algae. To confirm it, I measured the algal density and could observe that the average number of algal cells per host endodermal cell significantly reduced in the animals treated with antibiotics (**Figure 2.2.2C**). It could have been the result of an effect of loss of bacteria or a side-effect of antibiotic treatment.



**Figure 2.2.2.2: Effect of bacteria on the algal density**

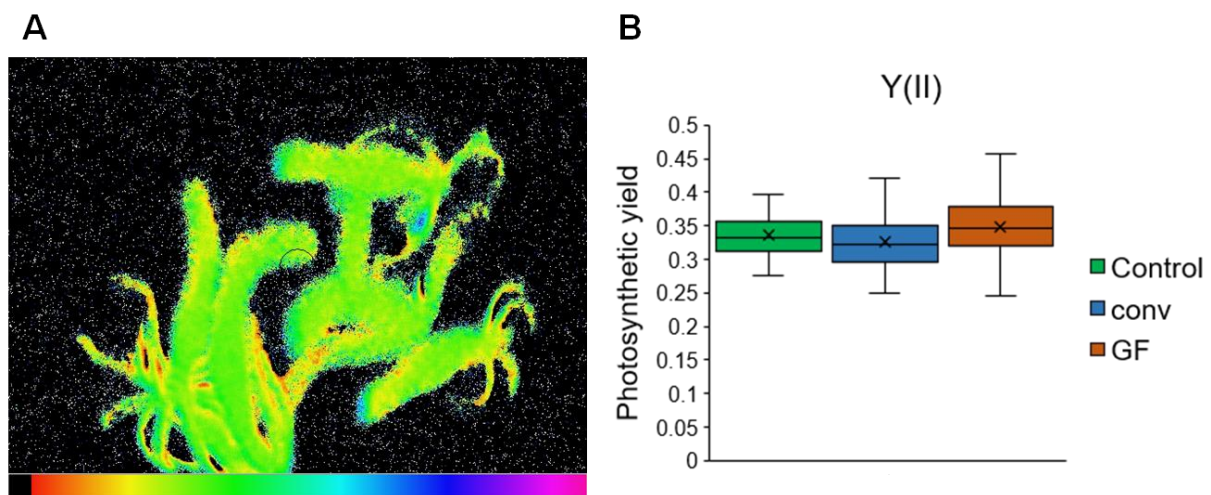
(A) Endodermal cells containing the algae were observed by cell maceration. (B) Timeline showing the antibiotics treatment and the point of sample collection for cell maceration after stopping the treatment. (C,D) Graphs showing the average number of algal cells per host endodermal cell after one month (C) and three months (D) of the antibiotics treatment. 250-300 endodermal cells were counted for the algal density per biological replicate (n=6, Unpaired Student's t-test, p-value \*\*\*p<0.001).



To disentangle the causative effect, animals were maintained under sterile conditions for two months with regular feeding with the germ-free *Artemia* before repeating the measure of algal density. It was observed that the algal density was restored back to the normal count similar to controls (DMSO and Conventionalized) animals. Hence it can be said that the observed reduction in the number of the algal cells was the result of the effect of antibiotics treatment and not an effect of the absence of bacteria (**Figure 2.2.2D**). Thus, bacteria seem not to have a direct and profound effect on the algae.

### ***In hospite* photosynthetic yield of the algae**

To gain further insight into the fitness of the algae in the germ-free animals, I measured the photosynthetic yield of photosystem-II (PSII) using imaging pulse amplitude modulation (PAM), and the parameter Y(II) was used to assess the working of the reaction center at PSII. In short, Y(II) (yield) represents the efficiency of the conversion of light energy to the excitation of the electron in the reaction center and its passage through the electron carriers.



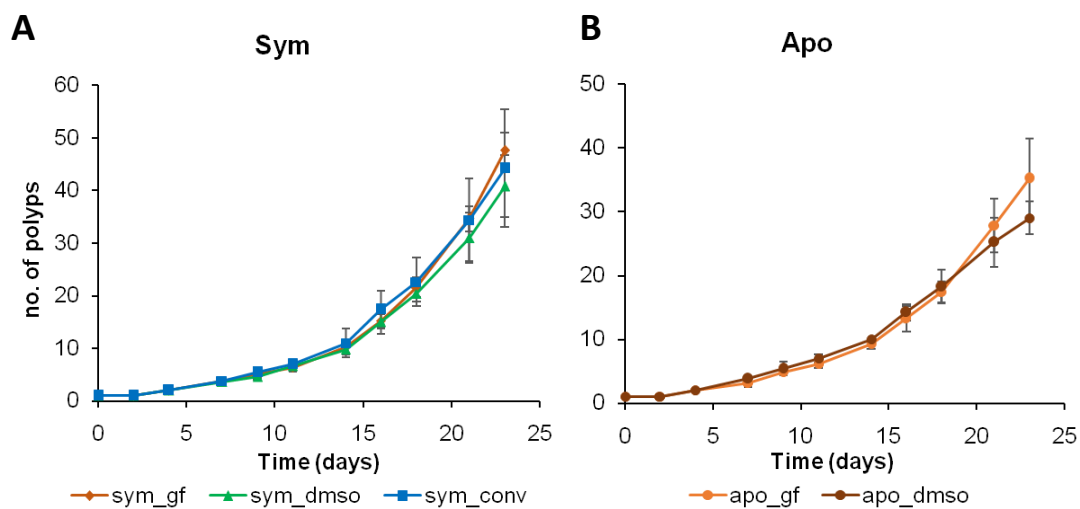
**Figure 2.2.2.3: Effect of bacteria on the photosynthetic yield**

(A)The image shows the output of Imaging PAM. The color bar at the bottom shows increasing Y(II) from red to pink (left to right). (B) The photosynthetic yield Y(II) levels of the algae *in hospite*. N = 14-16, Unpaired Student's t-test, non-significant.

I could observe that there is no significant difference in the photosynthetic yield between the germ-free and control animals (**Figure 2.2.2.3B**). This indicated that there is no direct effect of the bacterial symbionts on the algal photosynthesis.

## Population growth rate

A direct measure of host *Hydra* fitness is to assay its population growth rate. It has been successfully shown that disturbances in the tissue homeostasis, microbiome composition, or the environmental conditions result in alteration of the population growth trajectory (Habetha et al., 2003; Mortzfeld et al., 2019; Rathje et al., 2020). Moreover, as mentioned in chapter 2.1, the symbiotic animals show a reduced growth rate on the acquisition of the *Legionella sp.* bacteria.



**Figure 2.2.2.4: Population growth rates of the symbiotic and aposymbiotic animals fed three times a week**

The graphs show the population growth rate of the symbiotic (A) and aposymbiotic (B) animals. The animals were fed three times a week, N=5. There was no observable difference in the growth rates between the germ-free and control animals.

To assess the host fitness in the absence of bacteria, I observed the population growth rate of the polyps of symbiotic as well as aposymbiotic animals under different feeding regimes. All the animals were fed with the germ-free *Artemia* unless mentioned otherwise. In the first case, the animals were fed three times a week, and the growth was noted. Interestingly there was no difference in the growth rate of the germ-free animals as compared to the control animals, be it aposymbiotic or symbiotic animals (**Figure 2.2.2.4A/B**).

However, it was surprising that when the animals were fed once a week, I could observe that the growth rate of the germ-free animals was higher as compared to the DMSO and conventionalized control animals (**Figure 2.2.2.5A/B**). It was

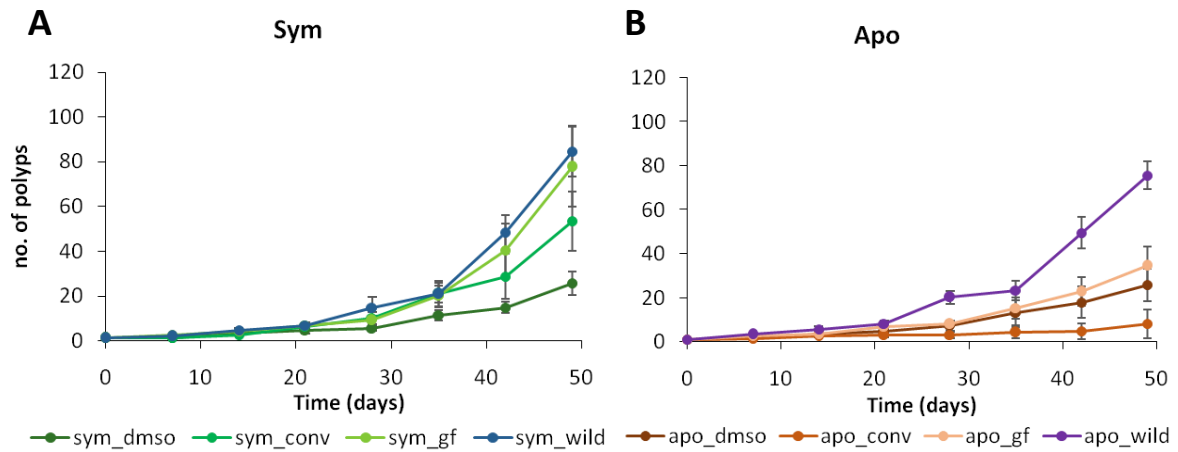
counter-intuitive, as opposed to the benefits, the bacteria were fitness costly for the host. However, I assumed that feeding the control animals with the germ-free *Artemia* would introduce some traces of antibiotics. These traces might alter the microbiome composition or alter the bacterial physiology, leading to a perturbed interaction with the host and hence a negative effect. 16S rDNA sequencing revealed that the microbiome composition of the DMSO and conventionalized animals indeed had some alteration in the microbial composition (**Figure 2.2.2.1B/C**) as compared to the wild<sup>†</sup> animals. However, this does not rule out the fact of altered bacterial physiology and might have an additive effect in reducing the host fitness. To address this assumption, I included the growth rate of the wild animals as an additional control, and they were fed with the wild-*Artemia* during the experiment. It is clear from **Figure 2.2.2.5A** that wild and germ-free symbiotic animals have similar growth rates that are higher than the DMSO and conventionalized controls. While in the case of the aposymbiotic animals, the wild animals have the highest growth rate.

There are two interesting observations made in this simple experiment:

- (i) The traces of antibiotics introduced through the germ-free *Artemia* have a profound effect on physiology in the case of DMSO and conventionalized animals. This effect can be accounted for the altered microbiome or the altered bacterial physiology on exposure to the antibiotics.
- (ii) If we compare the symbiotic and aposymbiotic growth rates in different bacterial backgrounds, it was observed that the symbiotic animals are less impacted by the above-mentioned effect. Rather, the aposymbiotic animals showed a clear reduction in fitness in the absence of bacteria and had a profound effect on the phenomenon mentioned in the previous point. These observations indicate the dominating and complementing role of the algal symbionts in the holobiont. In the absence of the stronger symbiont, algae, the role of bacteria becomes prominent for the host fitness.

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<sup>†</sup> Wild animals refer to the animals that were never treated with the antibiotics or DMSO solvent and were fed with wild *Artemia*.



**Figure 2.2.2.5: Population growth rates of the symbiotic and aposymbiotic animals fed once a week**

The graph shows the population growth rates of the symbiotic (A) and aposymbiotic (B) animals—N= 5 per treatment. The animals were fed only once a week with germ-free *Artemia* except for the wild-control.

### Response to starvation

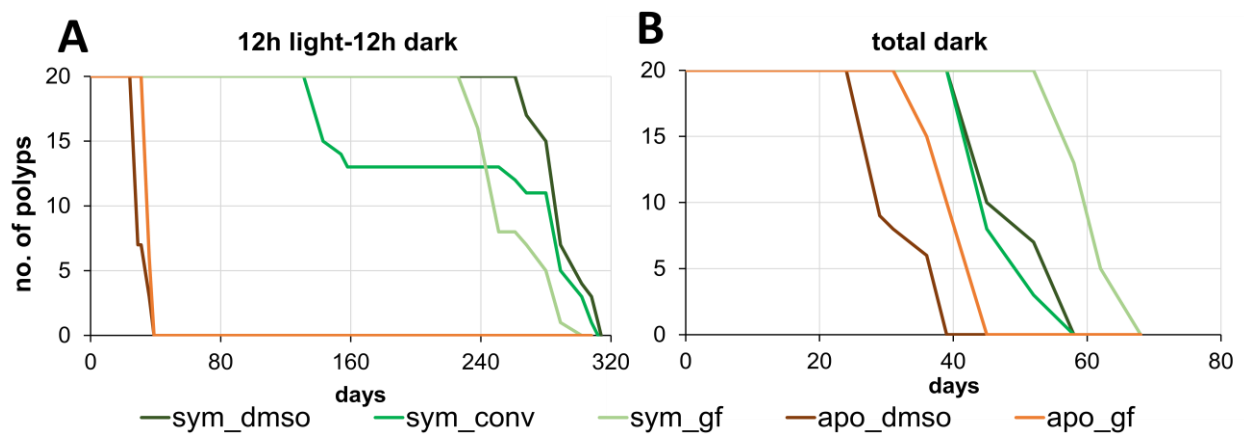
It has been observed in other *Cnidarians* harboring symbiotic algae, like corals, that the bacteria have a putative role in nutrient cycling and in some cases providing fixed nitrogen to the host (Glasl, Herndl, & Frade, 2016; Kelly et al., 2019; Rådecker et al., 2015; Wegley et al., 2007). Taking this into consideration, if the bacteria have a nutritional benefit, then it should be apparent under starvation conditions, and the animals with the bacteria will have a survival advantage.

To study this, the symbiotic and aposymbiotic<sup>‡</sup> animals, 20 polyps each of germ-free and controls, were subjected to a continuous starvation period, and the reduction in the population was monitored. The experiment was performed in both constant darkness and 12h-12h light-dark cycles.

The results from constant darkness were clear; the germ-free animals, both symbiotic and aposymbiotic, showed better survival under starvation as compared to their respective control animals (**Figure 2.2.2.6B**). This observation suggested that

<sup>‡</sup>The conventionalized animals control is missing for the aposymbiotic animals because they caught fungal infection repeatedly after roughly 2 weeks of starvation.

the bacterial symbionts are fitness costly for the host and provided no survival advantage whatsoever.



**Figure 2.2.2.6: Survival of the symbiotic/aposymbiotic animals under starvation**

The graphs represent the survival of the germ-free/control animals maintained under 12h-12h diel cycle (A) and under complete darkness (B) under total starvation.

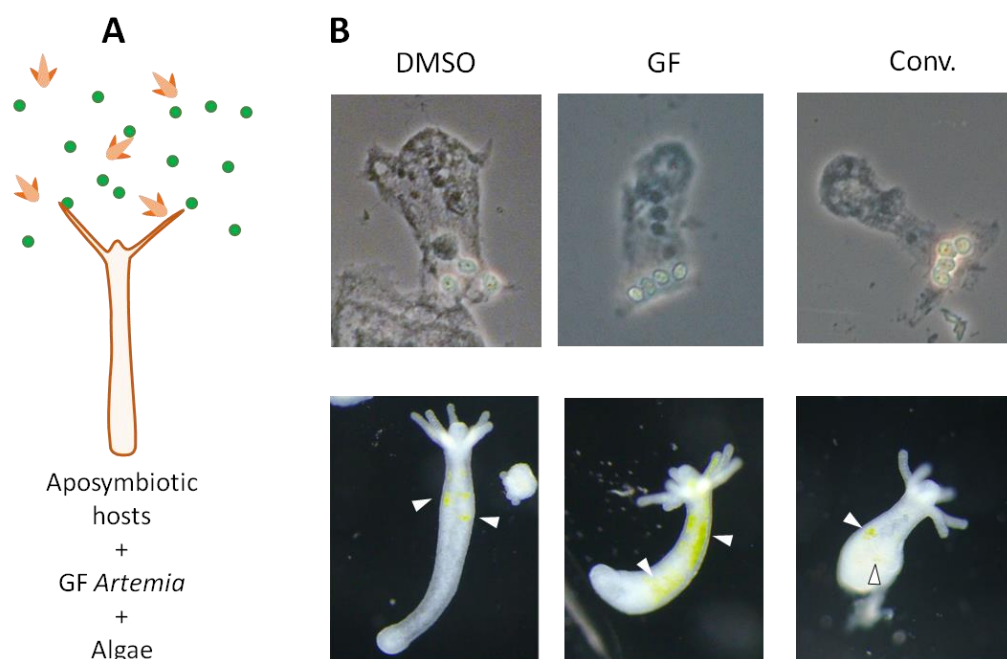
However, the results of starvation responses in the diel cycle are less clear. There is no major difference between treatments in the aposymbiotic animals. While, in the case of the symbiotic animals, the DMSO controls showed a tendency of better survival under starvation, but the conventionalized animals showed an unusual survival trajectory (**Figure 2.2.2.6A**).

Thus, there is an overall impression that bacteria might be beneficial to the symbiotic animals under prolonged starvation conditions. However, in order to accept these results, it is recommended to repeat this experiment and include wild-animal-controls and also with a bigger number.

### Symbiogenesis in aposymbiotic animals

If there exists an aposymbiotic *H. viridissima* host in nature, it will obtain the symbiotic *Chlorella* from the surrounding polyps via horizontal transfer. The algae have to enter the gastric cavity of the host and be taken up by the endodermal cells through phagocytosis (Davy et al., 2012). Once intracellular, the algae will repopulate the host and provide fitness advantage (Habetha et al., 2003). The crucial

step here is to have a successful establishment of the symbiosis or “Symbiogenesis.” It is less probable the bacteria residing in the ectodermal glycocalyx to have an effect on the uptake of the symbiotic algae by the endodermal cells. The cell-biology of the host and the symbiont has been very well characterized that are involved in this process (Bucher, Wolfowicz, Voss, Hambleton, & Guse, 2016; Davy et al., 2012; Weis, 2019). I, nevertheless, assayed the germ-free aposymbiotic animals for their ability to establish a successful symbiosis, hypothesizing that there might be potential secretory factors from the bacteria that would facilitate the uptake of algae by the aposymbiotic host.



**Figure 2.2.2.7: Effect of bacteria on symbiogenesis in the aposymbiotic hosts**

(A) The aposymbiotic hosts were fed with germ-free *Artemia* while placed in an algal suspension in sterile S-media. (B) The images show the successful establishment of symbiosis in all aposymbiotic hosts. The upper panel represents the cell macerates showing few algal cells at the base of the endodermal cells. The lower panels represent whole polyps of the respective treatment groups. The green spots in the lower panels, pointed by white arrow-heads represent successful symbiosis with the algae

To demonstrate the role of bacteria on symbiogenesis, I placed the aposymbiotic animals, germ-free, and controls, in sterile S-media suspended with *Chlorella A99*. These algae were freshly isolated from the germ-free symbiotic animals (**Figure 2.2.2.7A**). These animals were then fed with germ-free *Artemia*, to

enable them to ingest the algae along with the food. It should be noted that the animals were fed with minimal food, 1-2 *Artemia* per polyp. They were cleaned the next day and incubated in the sterile S-media for one week before checking the progress. At the end of one week, all three treatment groups showed clear signs of stable symbiogenesis (**Figure 2.2.2.7B**). Thus, it was further suggestive that the bacteria do not interfere with the symbiogenesis process and that the core cell biology of host and algae are solely responsible for the uptake of the algae.

### **Phototaxis in symbiotic animals**

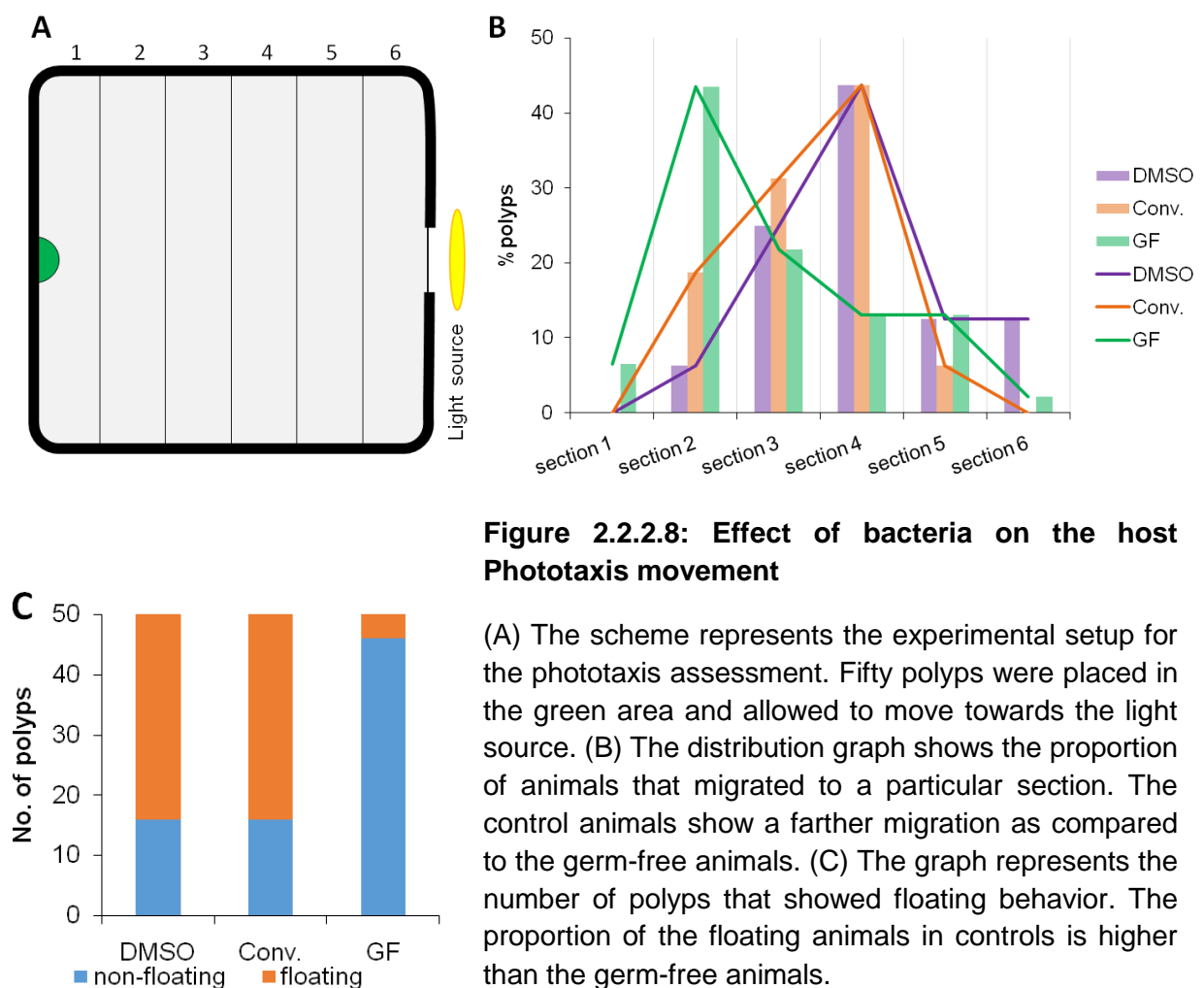
It is now a general knowledge that bacteria have a profound effect on the host behavior, be it a simple body-planned *Hydra* (Murillo-Rincon et al., 2017) or an organism as complex as mice and humans. It has been shown in *Hydra* that the bacteria can regulate the spontaneous body contractions or the squishing-behavior of the host (Murillo-Rincon et al., 2017).

Sir Trembley was the first to have observed the movement of the green *Hydra* towards the light in the 1740s. Despite the absence of a distinct light sensing organ or a tissue, *Hydra* can sense light, putatively through the homologs of the opsin protein (Macias-Munõz, Murad, & Mortazavi, 2019; Plachetzki, Fong, & Oakley, 2012). So I decided to assess if the phototactic movement is influenced by the bacterial symbionts. The following experiment is only a pilot experiment and needs to be repeated for a concrete conclusion.

A square plastic dish was divided into six parallel zones (**Figure 2.2.2.8A**), and a small area (in green) was marked in the first zone. In this green zone, 50 symbiotic polyps were placed, each for the germ-free and control animals and the plate was filled with sterile S-media. The entire plate was covered with the aluminum foil, except for a small slit on the opposite side of the green zone. This slit acted as a source of the light stimulus, and the over-head light was used as a light source. This was to control the amount of light entering the plate and have a unidirectional movement of the animals.

After one week of incubation, the position and state of the animals were noted. Interestingly two different behaviors were noted: floating behavior (Hetzler,

Rosenfeld, Ripeckyj, & Kosnik, 1975; Kepner & Miller, 1928) and forward movement. At the end of one week, not all the test polyps adhered to the bottom surface, a variable number of polyps managed to generate an air bubble at the foot region and started to float. The proportion of the floating animals in the case of the control animals (68%) was much higher (Figure 2.2.2.8C) as compared to the germ-free animals (8%). For the Phototaxis behavior, only the polyps adhering to the floor were taken into consideration. The control polyps (with modal section 4) migrated further than the germ-free polyps (with modal section 2) (Figure 2.2.2.8B). This portrayed the potential effect of bacteria on the behavior of green *Hydra*.



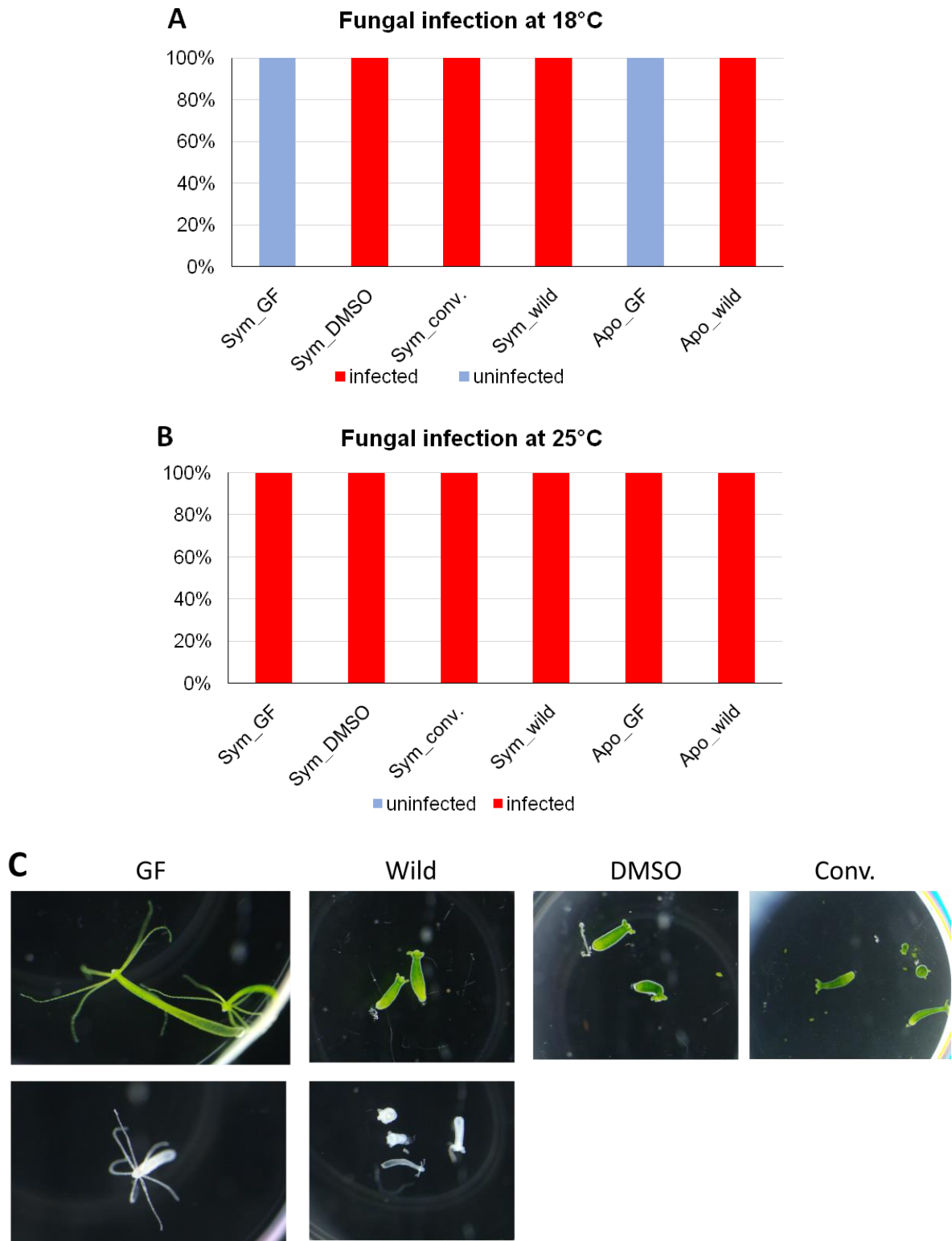
Although the results are interesting, as suggested earlier, this was a pilot experiment and needs to be repeated and also include aposymbiotic animals for comparison. The reproducibility of the results would suggest a strong effect of bacterial symbionts on light perception and/or the mobility of the animals.



## Resistance to pathogenic fungi

*H. vulgaris* AEP has been successfully studied for the role of bacterial symbionts in providing protection against pathogenic *Fusarium* fungi (see section 1.2). The bacteria appeared to provide less protection individually, but the additive and synergistic effect of two or more bacterial partners provide better protection against the fungi. So, in order to check if the bacteria on *H. viridissima* can provide similar protection to the host, I infected the polyps, symbiotic and aposymbiotic with different bacterial backgrounds, with the same *Fusarium* fungal spores and monitored the infection on the polyps. The original assay, as mentioned in (Fraune et al., 2015), involved treating the polyps with ~500 fungal spores per polyp and incubation for seven days. The readout was the germination and hence the appearance of the fungal fibers or not.

It was astonishing and counter-intuitive to observe that there had been no fungal growth in the germ-free animals, while in the case of the control polyps, the fungal spores showed evident germination and growth (**Figure 2.2.2.9A**). This only indicated that the bacteria are aiding in spore germination. This experiment was performed at 18°C that is the normal physiological temperature for *Hydra*. Moreover, as opposed to *H. vulgaris* AEP (Fraune et al., 2015), the fungus didn't tangle over the poly, and hence it was not a 'classical infection', nor was there any sign of stress over the host polyp. I repeated the same experiment combined with another stressor, temperature. The animals were incubated with the fungal spores at 25°C and monitored for the fungal growth and any alteration in host physiology. At the end of the incubation period, I could observe the fungal growth in all the treatments (**Figure 2.2.2.9B**). However, against all expectations, the control animals showed phenotypic degeneracy, while the germ-free polyps were healthy (**Figure 2.2.2.9C**). It has to be noted here that the fungal growth was not as observed in *H. vulgaris* AEP, i.e., tangles fibers over the polyp, but the fungal strands were growing homogeneously in the well.



**Figure 2.2.2.9: Role of bacteria in providing protection against fungal infection**

(A,B) The graph represents the proportion of animals where the fungal spores managed to germinate in germ-free and control animals at 18°C (A) and 25°C (B). The 'infection' refers to the germination of spores and the formation of visible fungal strands. (C) The images

represent the host phenotype at 25°C post fungal infection. The germ-free animals showed no sign of tissue degeneracy, while the control animals had a sick phenotype.

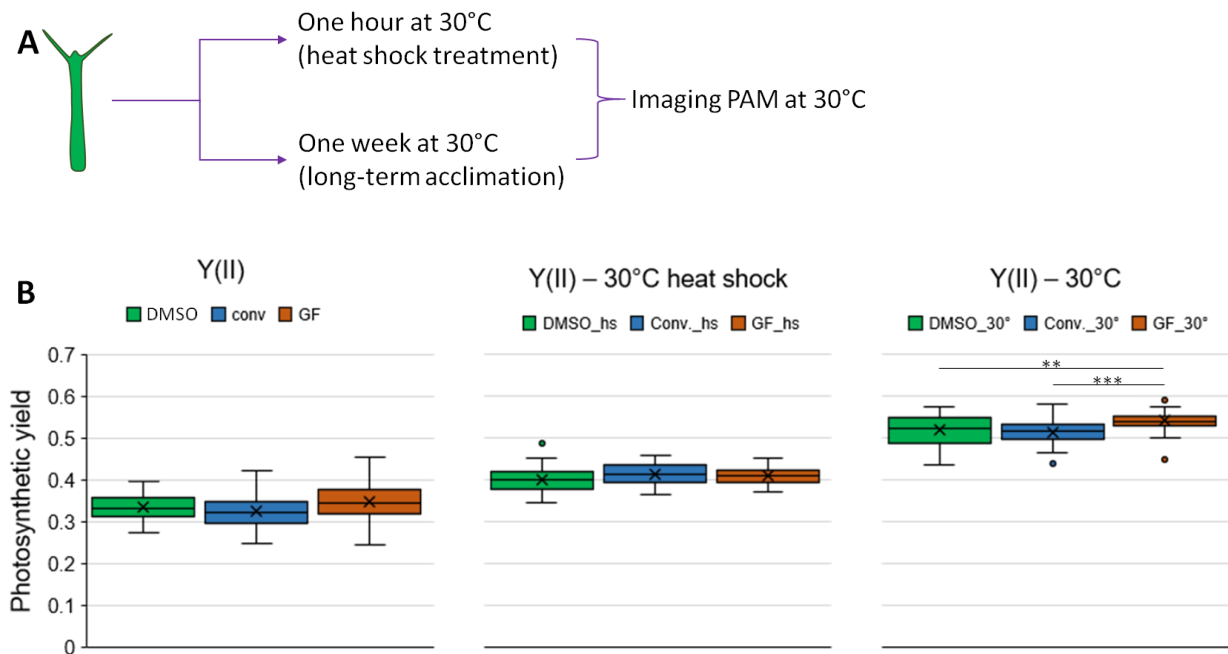
### **Effect of temperature stress on the holobiont physiology**

Apparent from the previous physiological assessment, it seems plausible that the bacteria might not have a direct influence on the host-algal symbiosis. However, this can hold true under normal physiological conditions, but probably not under the stress conditions. As observed in the previous experiment, even under the combined stress of a pathogen and temperature, the germ-free animals outperformed the control animals. So, I further decided to assess other physiological parameters under the temperature stress.

#### *Photosynthetic yield*

At 18°C, there was no difference in the photosynthetic yield of the algae and could observe no effect of bacteria whatsoever. However, heat resistant symbiotic bacteria can potentially provide temperature resilience to the host (Lima et al., 2020; Rosado et al., 2018; Ziegler et al., 2019; Ziegler, Seneca, Yum, Palumbi, & Voolstra, 2017). So, under long-term temperature stress, the bacteria should be able to adapt to the stress and provide resilience to the host as opposed to the germ-free animals. To study this, I incubated the symbiotic animals with different bacterial backgrounds at 30°C for two different time periods: one hour, for the heat-shock response and one week, for long-term acclimation. Followed by this, the photosynthetic yield (Y(II)) was measured at 30°C (**Figure 2.2.2.10A**).

There was a slight increase in Y(II) after one-hour heat shock treatment with no difference between the treatments (Figure 3.2.10B). After the long-term acclimation, Y(II) increased further to around 0.55 (**Figure 2.2.2.10B**). The Y(II) in germ-free animals was slightly higher than the control animals and also statistically significant. However, its biological significance is questionable, and the observed significance might be the result of a high replicate number (n=14-30).

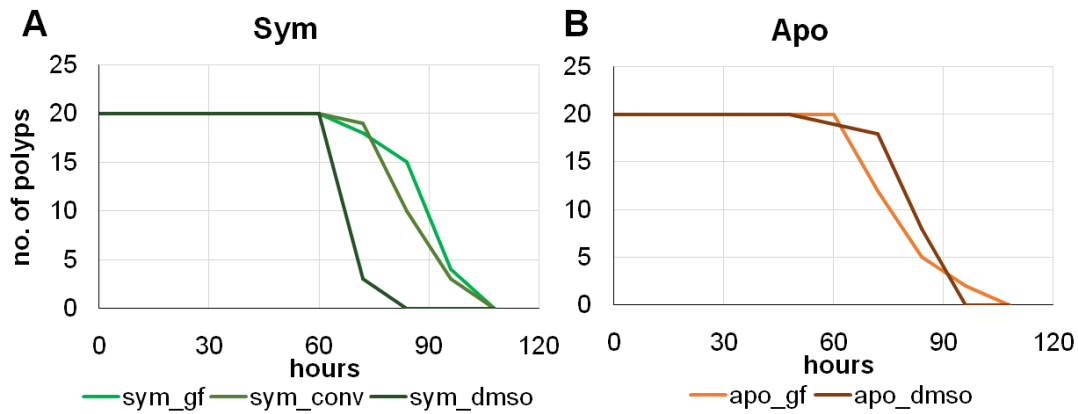


**Figure 2.2.2.10: Effect of temperature stress on the photosynthetic yield of the algae**

(A) The graphical representation of the experimental plan showing heat-shock and long-term acclimation. (B) The graphs represent the Y(II) levels of the algae *in hospite* followed by heat stress. The first graph represents the Y(II) at a normal temperature of 18°C. The second and third graphs represent the Y(II) levels after heat stress- heat-shock and long-term acclimation (Y(II)-30°C), respectively. (Unpaired Student's t-test, p-values: \*\*p<0.01, \*\*\*p<0.001)

### Temperature tolerance

The animals were subjected to a higher temperature stress 35°C, a lethal condition for the polyps. They were monitored for a reduction in the test population size. For, aposymbiotic animals, there was no considerable difference between germ-free and control animals. In the case of the symbiotic animals, the DMSO animals appeared to have a reduced resilience at 35°C as compared to the germ-free and conventionalized animals. These results still remain inconclusive, but by and large, the bacteria seem not to provide any temperature resilience to the holobiont. However, there is a limitation to this experimental setup. There is no pre-adaptation of the host-associated bacteria to higher temperatures. So, pre-conditioning of the animals at 30°C for an extended time period followed by lethal heat stress might portray the expected difference in stress resilience.



**Figure 2.2.2.11: Survival under temperature stress**

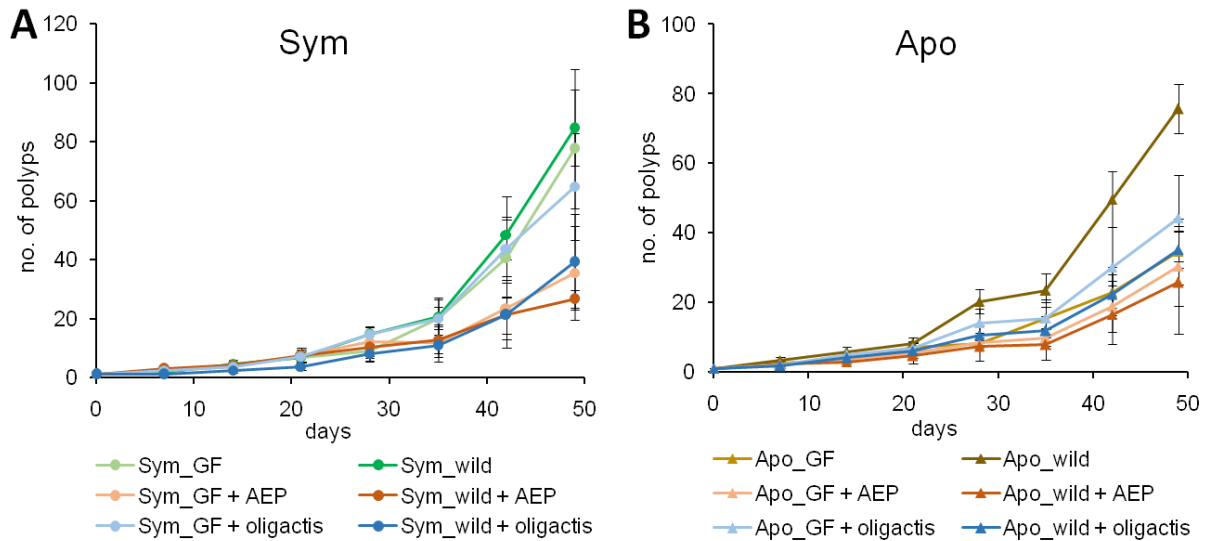
The graphs represent the survival of the symbiotic (A) and aposymbiotic (B) animals under lethal temperature stress. The animals were maintained at 12h-12h light-dark cycle at 35°C.

### Effect of the foreign microbiome on the host physiology

Since there was no effect of bacteria observable, I decided to replace the complete microbiome of the host with the entire microbiome from the other *Hydra* species. The symbiotic and aposymbiotic, germ-free and wild, animals were co-housed with wild *Hydra oligactis* or wild *Hydra vulgaris* AEP for two months and assessed for the microbiome composition and fitness. In the available time, I could only access the growth rate and survival under temperature stress.

#### Population growth rate

The population growth rate of the symbiotic and aposymbiotic animals, post two months of cross-species co-cultivation showed that in case of symbiotic animals, the wild animals (mono-cultivated), germ-free animals (mono-cultivated) and the germ-free animals co-cultivated with *H. oligactis* showed higher growth rate as compared to the other co-cultivation batches. In the case of the aposymbiotic animals, the wild animals out-performed all the other co-cultivated animals (**Figure 2.2.2.12**). This suggests that even the wild animals, with their complete microbiome, when co-cultivated with other species, showed a reduced fitness probably due to the horizontal transfer of bacteria.

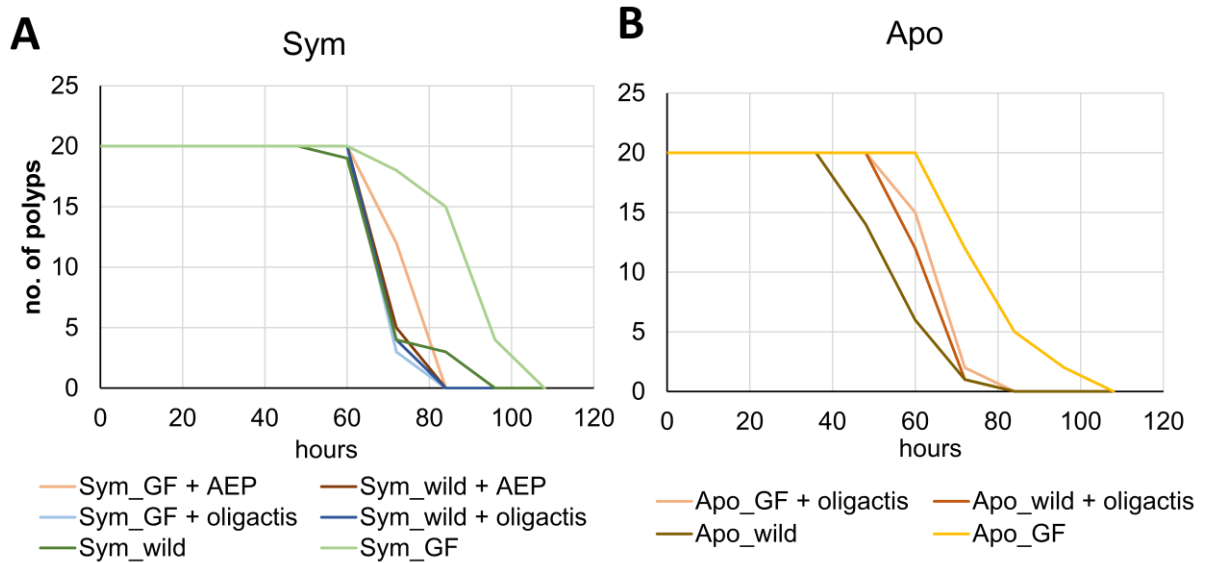


**Figure 2.2.2.12: The population growth rate of the symbiotic and aposymbiotic animals co-cultivated with *H. vulgaris* AEP or *H. oligactis***

(A) The wild and germ-free symbiotic, as well as the GF animals co-cultivated with *H. oligactis* animals, showed similar growth rates, which are higher than that of other co-cultivated animals. (B) In the case of the aposymbiotic animals, the wild animals showed the highest growth rate. In contrast, the GF animals, as well as all other co-cultivated animals, showed similar growth rates. (N=5)

### Survival under the temperature stress

As mentioned earlier, where the germ-free animals showed better survival at lethal temperatures, the symbiotic and aposymbiotic animals, germ-free and wild, co-cultivated with other *Hydra* species showed lower tolerance at lethal temperature dose. This indicates that the transfer of bacteria does not provide any temperature resilience in the case of symbiotic animals. In the case of the aposymbiotic animals, co-cultivation resulted in a slight improvement in the survival of the animals, however none comparable to the germ-free animals (**Figure 2.2.2.13**). (Note: the aposymbiotic animals co-cultivated with the *H. vulgaris* AEP could not be included in the experiment because, at the time of the experiment, they showed signs of severe tissue deterioration.)



**Figure 2.2.2.13: Survival of the symbiotic and aposymbiotic animals after cross-species co-cultivation**

The symbiotic animals (A) showed better survival without bacteria as compared to bacteria-harboring hosts. A similar trend was observed for the aposymbiotic animals (B), where the germ-free animals outperformed other treatments. However, the wild animals showed slightly lower tolerance as compared to the animals co-cultivated with the *H. oligactis* animals.

### 2.2.2.3 Discussion

To understand the interaction between the varied partners associated with the host has been the subject of interest since the discovery of symbiosis. It was further nurtured by the holobiont and hologenome concept introduced by Rosenberg and Rosenberg (Rosenberg, Koren, Reshef, Efrony, & Zilber-Rosenberg, 2007; Rosenberg & Zilber-Rosenberg, 2018; Zilber-Rosenberg & Rosenberg, 2008). Multi-partner symbiosis in corals has seen research of decades. In the swiftly changing Anthropocene and environmental conditions, the corals are facing a devastating loss of fitness that follows coral bleaching, thus generating a significant disturbance in the ecosystem. Therefore, it has become urgent to study the different interacting partners of the holobiont and attempt to improve their stability. However, the complexity of the corals and inaccessibility to study them functionally hinders the

overall progress from understanding the fundamental driving principles. With the technological advancement, the complexity of the corals started to unravel. Comparative genomics, transcriptomics, metagenomics, and microbiome studies have formulated several models that provide a working hypothesis concerning the interacting partners. But due to the experimental limitations, the models could never be tested empirically. So, the focus has been shifted to other model systems like *Aiptasia* (Ahmed, Herrera, Liew, & Aranda, 2019; Baumgarten et al., 2015; Lehnert, Burriesci, & Pringle, 2012; Rädicker et al., 2018) and *Hydra viridissima* (Habetha et al., 2003; Hamada et al., 2018; Ye, Badhiwala, et al., 2019) that harbors the symbiotic algae and a simpler microbiome (Fraune & Bosch, 2007; Röthig et al., 2016) to understand these fundamental interactions.

As mentioned in chapter 2.1, there is a profound effect of algal symbionts on the microbiome composition of *H. viridissima*. But, the other way round, the impact of the bacterial symbionts on the host-algal symbiosis was elusive. With the possibility to generate the germ-free animals, it was possible to elucidate the contribution of each partner. The work presented here was the first attempt to my knowledge to functionally address the contribution of different partners on the phenotype of the holobiont by looking elaborately at a wide array of phenotypes.

The experiments suggested no direct role of bacteria on the algal symbionts or the stability of its symbiosis with the host. Had there been any supplement of co-factors or small molecules from the bacterial symbionts that are vital for the algal photosynthesis, I would have observed its immediate effect on the algal photosynthetic yield and reduction in algal density in germ-free animals. If the contribution of the bacterial symbionts were nutritional, there would have been a definite effect on the survival response of the germ-free host to starvation. Any absence of such effects suggested uncoupled interaction between the bacteria and the algal symbionts.

Another evidence of uncoupled interaction between the bacteria and algae is the fact that the germ-free aposymbiotic animals could successfully establish symbiosis with the axenic algae. This portrays the involvement of the core host-cell-biology in symbiogenesis and the maintenance of symbiosis.



The population growth rate experiment hinted at an interesting phenomenon. Supplement of trace levels of antibiotics lead to changes in the microbiome composition and/or the bacterial physiology. It has been observed that very low levels of antibiotics are sufficient to alter the gut microbiota of mice (Roca-Saavedra et al., 2018), which might potentially lead to a diseased phenotype like obesity (Del Fiol, Balcão, Barberato-Fillho, Lopes, & Bergamaschi, 2018). In the case of *H. viridissima*, the antibiotics were introduced through the germ-free *Artemia* as they were prepared by antibiotics treatment (see **Materials and methods**). These were enough to reduce the growth of the control animals. It was further confirmed by the fact that the wild animals, fed with the normal *Artemia*, showed similar population growth as the germ-free animals. However, the most interesting observation was made in the aposymbiotic animals. Identical to the symbiotic animals, the animals fed with the germ-free *Artemia* had a reduced growth rate compared to the germ-free animals, but the wild animals fed with the normal *Artemia* showed the highest growth rate. This was the first evidence of the impact of bacteria on the host physiology. However, the effect was only visible in the absence of the algal symbionts.

A further effect of the bacteria was visible on the host physiology when they were assessed for the phototaxis movement. The fact that the bacteria have an impact on the host nervous system in *Hydra vulgaris* AEP and affect the spontaneous contraction (Murillo-Rincon et al., 2017), showed the effect of the bacterial symbionts on the host behavior. A similar phenomenon was observed in the germ-free green *Hydra*, that they showed reduced mobility as compared to the control animals. This points to the fundamental role of bacteria in modulating the host nervous system and hence the mobility, be it spontaneous contraction or the forward movement.

It is possible that the bacterial symbionts didn't show any effect under normal physiological conditions, but their contribution might be apparent under the biotic or abiotic stress condition. The biotic stress was provided in the form of the fungal pathogen. On the contrary to the observations made in *H. vulgaris* AEP (Fraune et al., 2015), the germ-free animals didn't show any sign of fungal germination under normal physiological condition, and the controls did. However, the 'classical-infection', where the fungal strands grow all over the polyp (Fraune et al., 2015), was not observed, and there was no tissue degeneracy observed. To dwell further, it was

combined with the abiotic temperature stress. At higher temperature, I could observe the spore germination in all treatments, with the tissue degeneration in the control animals. So, contrary to provide protection, the bacteria assisted the growth of the fungal pathogen.

Under the abiotic temperature stress, the photosynthetic yield of the algae was measured, yielding no significant result that might point to the temperature resilience provided by the bacteria. A similar observation was made when I explored for tolerance to the lethal temperature, where the germ-free animals outperformed the controls. However, the experimental design can be improved, and the animals can be pre-acclimated at sub-lethal temperatures before providing the lethal dose. The bacteria might likely adapt to the higher temperature and then aid in providing better protection at higher temperatures. A similar observation has been made in the context of the algal symbionts where the acclimated algae provided resilience at higher temperatures in green *Hydra* (Ye, Badhiwala, et al., 2019; Ye, Bhattacharjee, et al., 2019).

Since there were no direct and profound effects of bacterial symbionts on the algal symbionts observed, I went a step further and colonized the germ-free symbiotic and aposymbiotic animals with the cross-species microbiome. There have been several studies that involved the xenotransplantation of the human microbiome in mice and pigs and studied its effect physiology (J et al., 2011; Pang et al., 2007; Wrzosek et al., 2018). But the aim here was to transplant the cross-species microbiota so that to see if the massive alteration in the microbiome leads to an imbalance in the symbiosis between the host and the algae. The results were surprising because even the control animals that were co-cultivated with other species showed a reduction in holobiont fitness. It points to the possibility of horizontal transfer of bacteria. Moreover, the reduction in fitness of the co-cultivated animals suggests the importance of harboring native and stable microbiome composition for the host fitness.

There is a dominant symbiont in the green *Hydra* host that populates every endodermal cell. Every green *Hydra* strain shows phylosymbiosis with their respective algal symbionts (Schwentner & Bosch, 2015), like the species-specific phylosymbiosis to their respective microbiome (Franzenburg, Walter, et al., 2013).

Being intracellular, the algae face a harsher environment and lesser mobility to the surrounding environment, thus a more significant selection pressure. The result of this is a true co-evolution between the host and the algae that resulted in the loss of algal autonomy and genomic complementation (Hamada et al., 2018). These algal symbionts have been demonstrated to provide cross-generational temperature resilience to the holobiont (Ye, Badhiwala, et al., 2019). A similar phenomenon is hypothesized concerning the coral bacterial symbionts based on several correlational models (Lima et al., 2020). However, the results from green *Hydra* suggest so far that the strongly co-evolved algal symbionts can complement the bacterial functions by and large to maintain the holobiont stability. However, the effect of the bacterial symbionts becomes apparent only in the absence of the algal symbionts.

The results so far indicate that maintaining bacteria is costly for the host. Not harboring the bacterial symbionts seemed to be providing a competitive advantage to the symbiotic animals. However, this does not indicate the bacterial symbionts do not have any function on the host. The wild aposymbiotic animals had a superior growth phenotype over the germ-free aposymbiotic animals (**Figure 2.2.2.5B**). Simply colonizing the host surface can be protective against the invading bacterial pathogens and thus provide colonization resistance (Sorbara & Pamer, 2019). It is equally likely that I didn't look at the right phenotype or looking at the molecular profiles that might reveal the compensatory mechanisms in host and algae due to the absence of bacteria, ultimately stabilizing the phenotype. Taking this into consideration, it is suggestive that the contribution of the bacterial partners needs a careful and more in-depth study so that to reveal its effect on the functioning of the metaorganism.

### 3 Thesis summary and outlook

Host-associated bacteria have been a significant focus of research to understand their effect on the host physiology and behavior. The understanding of interactions in simpler animals aid in understanding the fundamental guiding principles that can be applied to higher organisms and their ecology. However, apart from these two partners, a metaorganism is comprised of other symbiotic eukaryotic and viral partners as well. Thus, for a complex metaorganism, it is essential to be able to separate these interacting partners to be able to understand the contribution of each.

*Hydra* has proved to be a successful model that has made it possible to be able to understand the contribution of individual bacterial partners. Furthermore, with the aid of stable transgenics in the host as well as the main bacterial colonizer (Wein et al., 2018), it is now possible to pin-point to the molecular interactions, by gain/loss of function studies that guide the symbiosis. Moreover, the recolonization of the host with different bacterial symbionts revealed the contribution of individual bacteria and the consortia as a whole on host physiology as well as inter-bacterial interactions.

Apart from bacterial symbionts, *H. viridissima* also harbors endosymbiotic algae that show a robust metabolic interdependence with the host. As shown in chapter-1, these interactions are precise, where the maltose secreted by algae induces the expression of the symbiosis specific glutamine synthetase, rather than eliciting a generic metabolic response.

Moreover, the results presented in this thesis were the first attempt to functionally disentangle the interactions between the three partners- host, bacteria, and algae. The contribution of each partner could be accounted for because of the possibility of obtaining a host with different symbiotic backgrounds in terms of algae and bacteria.

In a metaorganism, the symbionts interact with the host as well as other symbionts. Similarly, in a multipartite organism like green *Hydra*, there might occur the interactions between the endodermally intracellular algae and the ectodermal extracellular bacteria. To explore these interactions, the hosts with different

symbiotic backgrounds were generated and assessed for the contribution of each partner. The results have been quite intriguing.

There was a clear effect of algal symbionts on the bacterial composition and controlling the invasive species. However, there was no distinct effect of the bacterial symbionts on the host colonized with the algae. The only concrete and observable difference were on the aposymbiotic animals, where the wild animals had a growth advantage over the germ-free animals. This suggests that a more robust and co-evolved algal symbiont might be complementing the effect of bacteria. The other observable difference was on the host behavior, where the absence of bacteria leads to reduced host mobility. Thus the bacteria portray the possibility of interactions with the host, which might, in turn, affect the algal symbionts.

Any phenotypic effect of bacteria on the algae was not observable based on the measured parameters. However, the alga probably has a coping mechanism to maintain the physiological stability of the holobiont. Thus it becomes prudent to check the transcriptional profile of the algae and the host in the absence of bacteria to have a mechanistic understanding of this tripartite interaction.

It was also probable that the bacterial symbionts were beneficial under the stress conditions. Surprisingly, there was no distinct benefit of harboring bacteria under the stress condition. The biotic stress was given in the form of fungal infection, but no discernable effect of harboring bacteria was observed unless it was combined with the temperature stress. Harboring bacteria proved to be detrimental for the host, and the control animals showed the diseased phenotype. Abiotic stress was applied in the form of temperature stress. Repeatedly there were no beneficial effects of the bacteria observed.

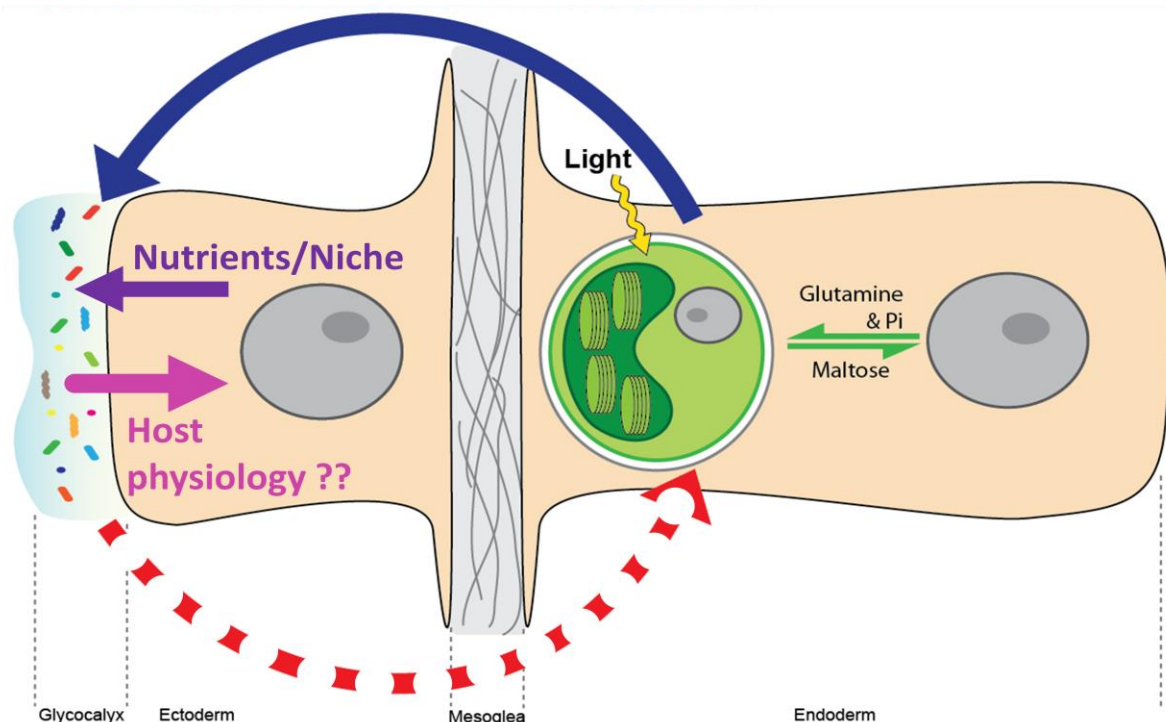
It has to be noted that the lethal temperature stress was applied to observe the temperature resilience of the holobiont prior to sub-lethal temperature acclimatization. It is known that the pre-acclimatization of the symbionts to a sub-lethal temperature provides better protection at lethal temperatures (Ye, Badhiwala, et al., 2019). So, as an outlook, the pre-acclimatization of the symbionts or holobionts at a sub-lethal temperature might result in better survival of animals at lethal temperatures. If the improved resilience is not observable on the germ-free

animals, it provides a clear indication that the bacterial symbionts can aid in improving resilience to abiotic stressors.

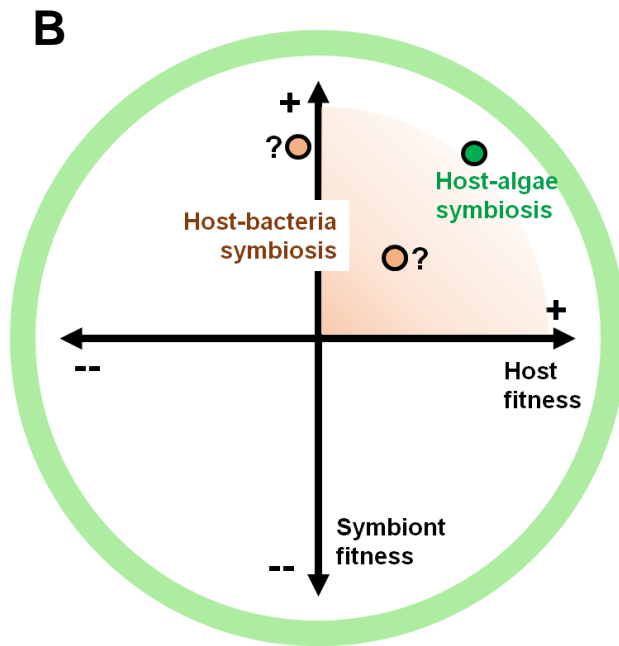
The observations presented in this thesis indicate that the bacterial symbionts occupy the niche on the host glycocalyx and utilize the nutrients secreted by the host (**Figure 3.1A**). The composition of these bacterial symbionts is modulated by the algal symbionts. However, due to the absence of any direct effect on the symbiotic host physiology, the exact nature of the bacterial symbionts, being commensal or mutualistic, is questionable. Therefore, the absence of algal symbionts might reveal the functional potential of bacteria on host physiology, which can be mechanistically studied by high-throughput methods.

If we plot a graph of host fitness vs. the symbiont fitness, the apparent position of the 'host-algal symbioses' will be in the first quadrant, where both the partners are mutually benefitted (**Figure 3.1B**). However, the absence of any effect of bacteria in *Hydra* hosts colonized with the algal symbionts, and their growth-promoting role in aposymbiotic animals suggests the possible position of 'host-bacterial symbioses' in first or second quadrant (**Figure 3.1B**).

## A *Chlorella* affects microbiota



**No direct effect**



**Figure 3.1: Schematic representation of the observed tripartite interactions**

(A) The endodermally located endosymbiotic algae affect the microbiome composition. However there is no effect of bacteria on the algae. There is a definite niche occupation and nutrient utilization but no discernable feedback on the host except in aposymbiotic animals. (B) Graphical representation of the host vs. symbiont fitness showing the status of the host-algal and host bacterial symbioses based on current understanding.

Cross-species microbial colonization of the host revealed the importance of harboring the native microbiome. This also represented an infection model as in *H. oligactis* (Rathje et al., 2020) that can be further explored to address the effect of an invasive or parasitic bacterium on the existing community profile and the fitness of the metaorganism. For the cross-species transplantation, the 16S rDNA data for the community composition needs to be incorporated to gain a better understanding of the transferred microbes and their effect.

With the technological advancements in generating high throughput data and modeling approaches, it has been possible to predict the potential interactions between different partners of the metaorganism. However, these predictions need to be substantiated with appropriate experimental proofs (He et al., 2019). Green *Hydra* has proven to be a successful experimental model that can be manipulated considerably under laboratory conditions and presents a possibility to assess a wide array of phenotypic properties, as mentioned in this thesis. Further implementation of high-throughput techniques holds the potential to reveal the fundamental forces that regulate these symbiotic interactions that can be extrapolated to a wider group of multipartite metaorganisms that are ecologically urgent to be studied like corals.

## 4 Materials and methods

### Animal husbandry

Experiments were carried out with the Australian *Hydra viridissima* strain A99, which was obtained from Dr. Richard Campbell, Irvine. In the study, the symbiotic hosts refer to the hosts with algal symbionts, and the aposymbiotic hosts refer to the same animals without algal symbionts. However, both the lines of animals contain bacterial symbionts. Aposymbiotic polyps were obtained by photobleaching using 5  $\mu\text{M}$  3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) as described before (Habetha et al., 2003) and subsequently cultivated for more than one year prior to the start of the experiments. All animals were cultivated at constant temperature (18 °C), light conditions (12 h / 12 h light/dark rhythm) and culture medium (0.28 mM  $\text{CaCl}_2$ , 0.33 mM  $\text{MgSO}_4$ , 0.5 mM  $\text{NaHCO}_3$  and 0.08 mM  $\text{KCO}_3$ ) according to the standard procedure (Lenhoff & Brown, 1970). For the co-housing experiments, symbiotic and aposymbiotic animals were co-cultivated and fed three times per week with freshly hatched *Artemia salina* for four weeks before DNA isolation. Before gDNA extraction polyps were starved for 72 h.

### DNA extraction

For each biological replicate (n=6) combined hydra and bacterial DNA was isolated from ten polyps. Animals were washed three times in sterile filtered (0.2  $\mu\text{m}$ ) culture medium and subsequently ruptured in buffer ATL (Qiagen) by using a 23G syringe or vortexing. Algae were removed by centrifugation; suspensions were centrifuged three times at 350 g for 2 min. After each centrifugation step, supernatant was transferred to new tube. The gDNA was extracted from the third supernatant using the DNeasy Blood & Tissue Kit (Qiagen) with elution performed in 50  $\mu\text{l}$  AE buffer. The extracted DNA was stored at -20 °C until sequencing.

### 16S rRNA gene sequence analysis

16S rRNA gene amplicon sequence analysis was conducted using the Qiime 1.8.0 package (Madigan, Cox, & Stegeman, 1984). Using the sequence fasta-file, a quality file and a mapping file, which assigned the 10 nt barcodes to the corresponding



sample as input, the sequences were analyzed using the following parameters: length between 300 and 400 bp, no ambiguous bases and no mismatch to the primer sequence. Chimeric sequences were identified using Chimera Slayer (Haas et al., 2011) and verified manually before removal from the data set. Putative chimeric sequences, which were present in at least two independent samples, were retained. Sequences were rarified to the lowest number of reads in the dataset, which was 2300 reads. Subsequently, sequences were grouped into operational taxonomic units (OTUs) at a  $\geq 97\%$  sequence identity threshold and mapped against SILVA database (Yarza et al., 2008).

### **Migration assay**

As shown in the **Figure 2.2.1.3A** 1ml syringes were filled with 1 ml of sterile water. In the treatment group, 10 symbiotic polyps were placed in each syringe. These syringes were placed vertically in 1 liter of water from the aposymbiotic animals ("source pool"). This source pool contained aposymbiotic animals for 1 week before the start of experiment. The syringes were incubated for 48 hours and the water in them was carefully collected and used for DNA extraction and quantification.

### ***Legionella* sp. quantification using real time-PCR**

*Legionella* specific primers were designed using the V1-V2 region sequences obtained from 16S rDNA sequencing; forward 5'-CTCTCAGACCAGCTACCGAT-3' and reverse 5'-TACTAGATGGGTGGCGAGTG-3'. They were checked by aligning against the RDP database using RDP probe (Cole et al., 2014) and against the 16S library of *H. viridissima* to avoid any non-specific amplification.

For the migration assay, 1 ml of water was collected from the syringe or the source pool (**Figure 2.2.1.3A**) and centrifuged at 20,000 rcf for an hour and a half. 950  $\mu$ l of the supernatant was removed away to avoid any disturbance in the pallet. DNA was isolated from the remaining 50 $\mu$ l water+pallet using DNeasy Blood & Tissue Kit (Qiagen) with elution performed in 25  $\mu$ l AE buffer. Real-time PCR was performed on 1 $\mu$ l of the template DNA using GoTaq qPCR Master Mix (Promega, Madison, USA) and ABI Prism 7300 (Applied Biosystems, Foster City, USA). The qPCR was performed in duplicate with six biological replicates each.

## **Algal expulsion quantification**

For “early co-housing”, mono-housed symbiotic polyps were co-housed with mono-housed aposymbiotic polyps for 48 hours prior to start of experiment. For “prolonged co-housing”, the animals were co-housed for one month. At 0 hours before the start of experiment, four symbiotic polyps of mono-housed animals and four polyps each of symbiotic and aposymbiotic animals, each of early/prolonged co-housing, were incubated in 1 ml of sterile culture water. After 24 hours, 900µl of water was carefully collected and centrifuged at 14,000 rpm for 5 min to pellet the algae. 850 µl of the supernatant was carefully removed away and the pellet was resuspended in the remaining 50 µl of water. It was immediately subjected to counting using flow cytometry on a BD FACSCalibur with CellQuestPro v5.2 (*Becton–Dickinson*) with a blue laser at 488 nm using forward scatter and FL3 filter (>670 nm). The data was further analyzed with FCSalyzer 0.9.13-alpha (<https://sourceforge.net/projects/fcsalyzer/>).

## **Generation of germ-free animals**

After several attempts to generate the germ-free animals, aforementioned is the most successful attempt. The animals were treated with antibiotic cocktail that was comprised of 1.2 mg/ml each of ampicillin, streptomycin, neomycin and rifampicin and 1.44 mg/ml of spectinomycin. The antibiotic solution was replaced daily. The antibiotic solution was carefully decanted without removing the animals, to avoid any damage. They were fed after one week of treatment with germ-free *Artemia* (He *et al*, unpublished). Next day of feeding, the animals were placed in a fresh antibiotic solution and a new container. The process was continued for 6 weeks. The sterility of the animals was checked initially by plating a meshed polyp on R2A agar plate twice a week followed by PCR based confirmation. 16S 27F-1193R primer set (Beckers *et al.*, 2016) was used for the amplification of the bacterial 16S rDNA gene (**Figure 2.2.2.1A**). However, for the germ-free symbiotic animals, additional confirmation was made by 16S rDNA sequencing. The results showed maximum 150 bacterial reads out of average 50,000 reads.

## **Generation of germ-free *Artemia* (He *et al*, unpublished)**

Freshly hatched shrimps were carefully isolated from the cysts' debris and washed with sterile sea water on a sieve. They were then placed in 25 ml volumes in a 50 ml tube containing the shrimps in antibiotic cocktail prepared in sterile sea water: 1mg/ml each of ampicillin, streptomycin, neomycin and rifampicin and 1.2 mg/ml of spectinomycin. The tubes were placed on a roller at 4°C. The antibiotic solution was changed every day and the shrimps were placed in a new tube. The process was continued for four days. On the fifth day, the antibiotic solution was replaced by sterile sea water. The shrimps were left without antibiotics for at least 24 hours before using them for feeding.

### **Generation of fungal spores and infection experiments**

The fungi, *Fusarium sp.*, obtained from *H. vulgaris AEP* (Fraune et al., 2015) were grown in liquid R2A media at room temperature for 48 hours. The suspension was passed through a sieve to separate out the bigger fungal filaments. The resultant turbid suspension was centrifuged at 10,000 g for 10 min. The resultant pellet was resuspended in sterile S-media with the final density of ~1000 spores per ml.

This spore suspension was used for the infection assays. 2 polyps were placed in a well containing 0.5 ml of spore suspension and incubated for 7 days at 18 or 25 °C. The final readout was the germination of spores and visible filaments in the well and around polyps.

### **RNA extraction and cDNA synthesis**

For each biological replicate, ~50 *Hydra* polyps were pooled together for RNA extraction. Total RNA of *Hydra* was extracted using the Trizol reagent and PureLink RNA Mini Kit (Thermo Fisher Scientific) according to manufacturer's instructions. cDNA was synthesized using First Strand cDNA Synthesis Kit, ThermoFisher using poly-T primers according to the manufacturer's protocol.

### **qPCR of the target metabolic genes**

Real-time PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, USA) and ABI Prism 7300 (Applied Biosystems, Foster City, USA). All qPCR experiments were performed in duplicate with three biological replicates each. Values were normalized to the expression of tubulin alpha, actin and elongation-

factor-1a genes. Primers used for the quantification are mentioned in the **Supplementary table 1**.

### **Counting of avg. number of algal cells and cell maceration**

Cell maceration was performed using 7% solution of 1:1 acetic acid:glycerol solution prepared in S-media. 2-3 polyps were placed in this solution for 15 min at room temperature and then lightly tapped to disintegrate the polyps (David, 1973). They were then fixed with equal volume of 4% PFA and spread on gelatin coated slides with Tween-20 detergent. They were dried overnight at room temperature and used for counting the algal density later. 200-250 cells per biological replicate were counted with 6 biological replicates for each treatment.

### **Imaging PAM for the measurement of photosynthetic yield (Y(II))**

Imaging-PAM, Walz Ltd., was used to measure Y(II) of the algal symbionts in the hosts. To reduce the mobility of the animals, they were embedded in 2% alginate prepared in S-media. The animals were starved for one week before taking the measurements to avoid any influence of nutrition from an external source.

## 5 Supplementary information

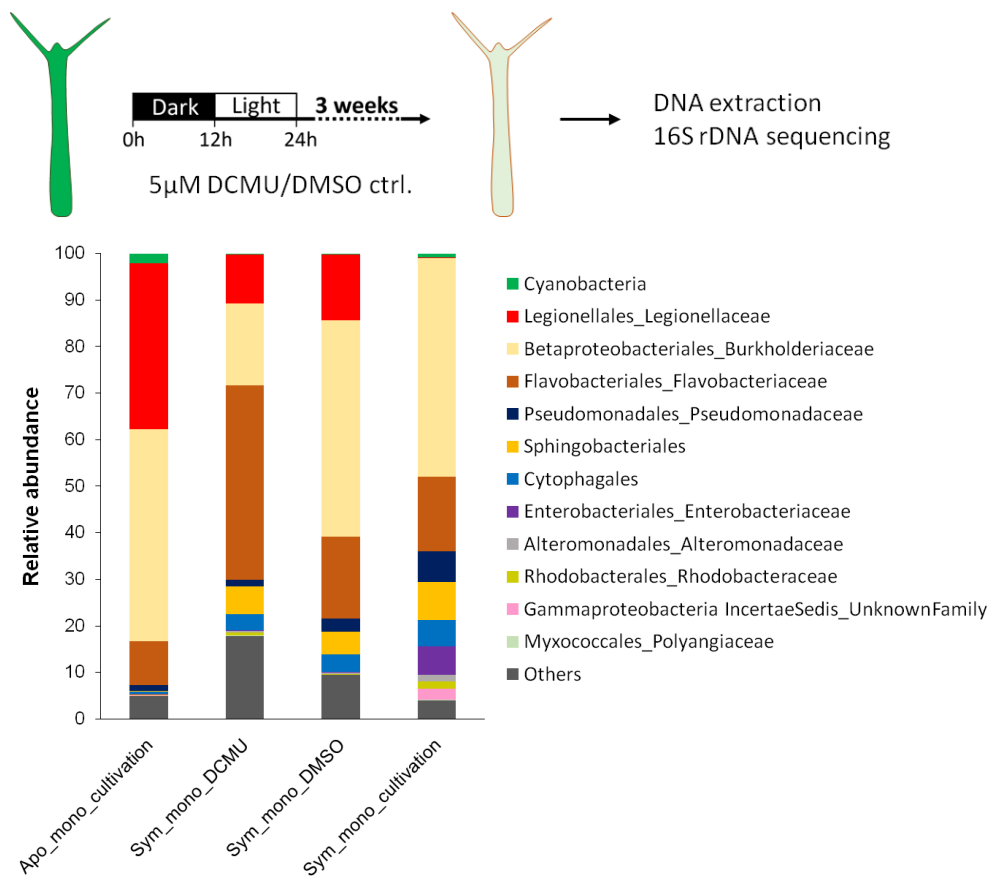
**Supplementary table 1: list of genes and corresponding primer sets**

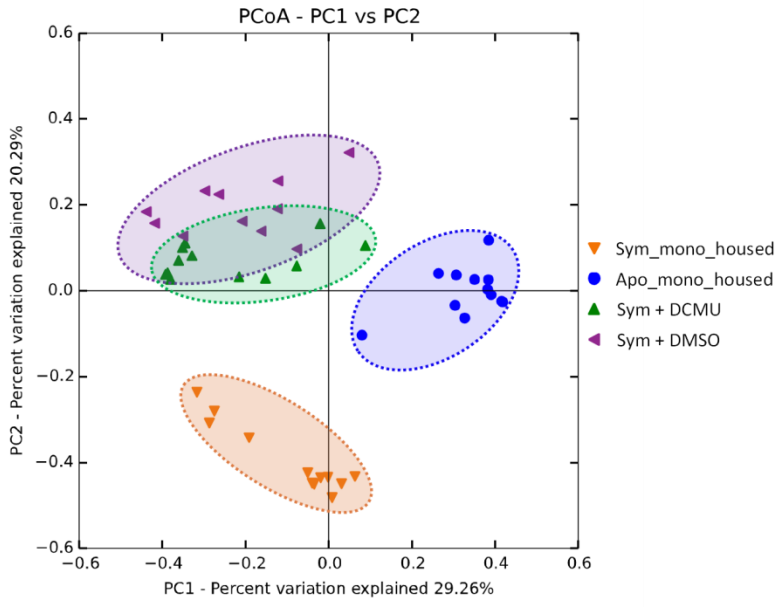
Gene	Forward (5'-3')	Reverse (5'-3')
GS-I	GTGGGTTGCTCGTTATCTACT TG	CACCAGGGATGGGTTTAGG
GS-II	GAAATGCGAAAAGAGGGCGG	AGTCGAAGTTTCGTGATGGC
GS-III	CCAGCGATTTTCAACCCTGC	TGGACATCCTCTTGGTTGGC
Spot-14	TTAAACTTCGAAGCTGGAAT GG	TTAGCGAAGACTTTGTCGTATG G
NaPi	TGTACCTGTCCATGGAATTAA AGC	TACCTTGTCCGAATAGCAGCTC
a-tubulin	TTCCTTGCTCATGGAACGAC	AGCAGGGTAAACTGCAAACCTCC
Elongation factor 1a	ACCAACATTGTCACCTGGGAG	GGAACAGTACCTGTTGGTCGT
16S 27F-1492R	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT
16S 27F-1193R	AGAGTTTGATCCTGGCTCAG	ACGTCATCCCCACCTTCC
<i>Legionella sp.</i> 16S	CTCTCAGACCAGCTACCGAT	TACTAGATGGGTGGCGAGTG

## Supplementary information 1:

### Conversion of the symbiotic animals to aposymbiotic animals

If the aposymbiotic animals were able to inhabit the *Legionella* bacteria, it was likely that conversion of the symbiotic animals to the aposymbiotic ones would bring up the rudimentary population of the *Legionella* bacteria in the host to a higher level. To check this, I treated the symbiotic animals with 5µM DCMU, a photosynthesis inhibitor and the corresponding concentration of DMSO for a solvent control. The animals were incubated for 3 weeks in the diel cycle (**Figure A.1.1A**) before DNA extraction. 16S rDNA sequencing revealed that the treated as well as the control animals showed an increased abundance of *Legionella sp.* So, it becomes intangible to comment if the photosynthesis inhibitor itself showed an effect as the solvent itself had a profound effect on the microbiome composition.

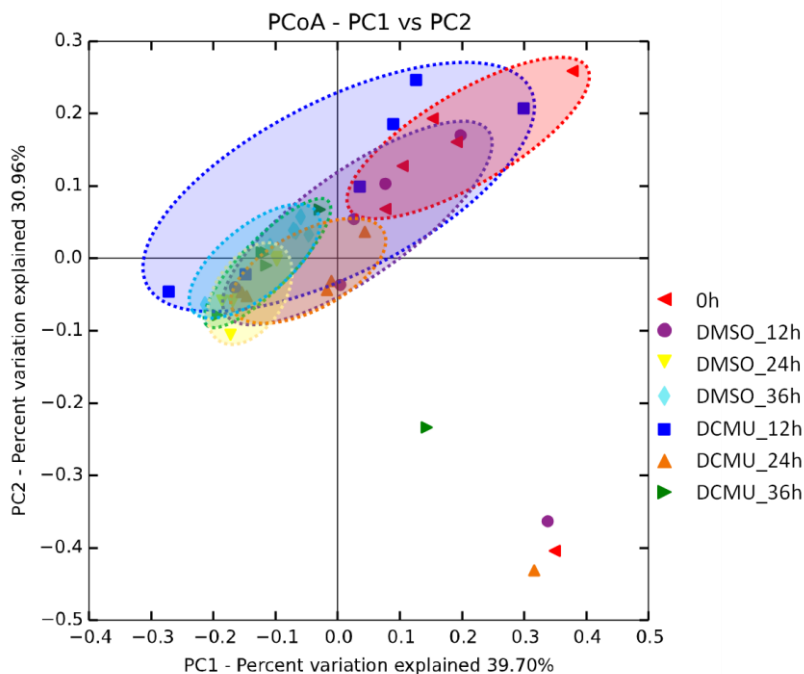
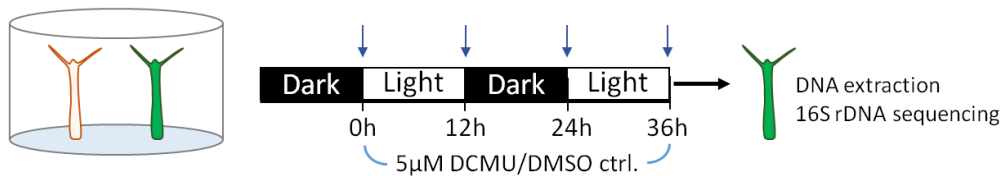




## Supplementary information 2:

### Inhibition of the Day-night cycling with the photosynthesis inhibitor

Since, the microbiome shifts are observed in the co-cultivated animals which is subjective to the light conditions, I hypothesized that blocking the algal photosynthesis without altering the light conditions would result in the blocking of the microbiome shifts. So, the animals were treated with 5 $\mu$ M DCMU and corresponding DMSO concentration in the solvent control (**Figure A.1.2A**). The samples were collected after the dark and light periods (**Figure A.1.2A**) and DNA was isolated from the symbiotic animals. 16S rDNA sequencing revealed that the periodic shifts in the microbiome composition stopped in both, the DCMU treated as well as the solvent (DMSO) control animals. Once again the solvent masked the effect of the actual test compound and the results remained inconclusive.





## 5 List of publications

### Scientific article

Mayuko Hamada, Katja Schröder, Jay Bathia, Ulrich Kürn, Sebastian Fraune, Mariia Khalturina, Konstantin Khalturin, Chuya Shinzato, Nori Satoh, Thomas CG Bosch, Metabolic co-dependence drives the evolutionarily ancient *Hydra–Chlorella* symbiosis, eLife 2018;7:e35122 DOI: 10.7554/eLife.35122

### Review and perspective

Esser D, Lange J, Marinos G, Sieber M, Best L, Prasse D, Bathia J, Rühlemann M, C, Boersch K, Jaspers C, Sommer F: Functions of the Microbiota for the Physiology of Animal Metaorganisms. J Innate Immun 2019; 11:393-404. doi: 10.1159/000495115

He, J., Lange, J., Marinos, G., Bathia, J., Harris, D., Soluch, R., Vaibhvi, V., Deines, P., Hassani, M. A., Wagner, K., Zapien-Campos, R., Jaspers, C., Sommer, F., Advancing Our Functional Understanding of Host–Microbiota Interactions: A Need for New Types of Studies. BioEssays 2020, 42, 1900211. doi:10.1002/bies.201900211

### In press (book chapter)

Jay Bathia, Thomas CG Bosch, Symbiotic interactions in the holobiont *Hydra*, book chapter, CRC publishing house “*Cellular Dialogues in the Holobiont*”, in press

### In preparation

Jay Bathia, Katja Schroeder, Sebastian Fraune, Philip Rosenstiel, Thomas CG Bosch, Symbiotic algae of *Hydra viridissima* play an active role in shaping the host microbiome and controlling microbial colonization, in preparation

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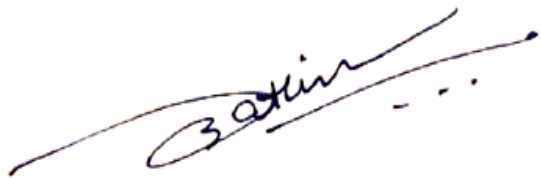
And the one.

## 8 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis eigenständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater Prof. Dr. Dr. h.c. Thomas C. G. Bosch in Anspruch genommen. Des Weiteren erkläre ich, dass ich noch keinen Promotionsversuch unternommen habe.

Teile dieser Arbeit wurden bereits zur Publikation eingereicht oder veröffentlicht.

Kiel, den 01.06.2020

A handwritten signature in blue ink, appearing to read 'Jay Bathia', written over a horizontal line.

Jay Bathia