The response of the Baltic Sea sponge *Halichondria panicea* upon challenge with *Vibrio* bacteria

Masters Thesis

submitted by

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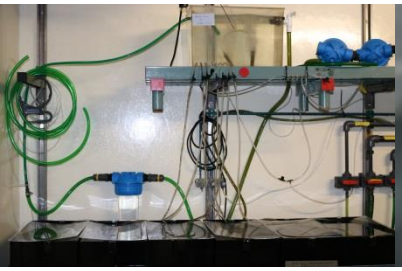

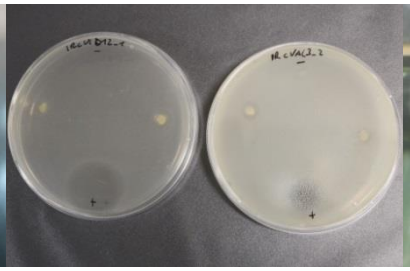


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Content

1. Zusammenfassung

Schwämme (Stamm Porifera) nehmen über ihre filtrierende Ernährungsweise ständig Mikroorganismen auf, darunter auch potentielle Pathogene, während sie zeitgleich spezifische mikrobielle Gemeinschaften beherbergen. Es ist noch nicht bekannt wie Schwämme die unterschiedlichen Mikroorganismen voneinander unterscheiden (Symbionten, Nahrung, Pathogene). Ich stellte die Hypothese auf, dass das „angeborene“ Immunsystem des Schwamms eine Rolle spielen könnte, um eine spezifische Reaktion gegenüber den Mikroorganismen hervorzurufen. Darüber hinaus ist die Rolle des „angeborenen“ Immunsystems in Bezug auf das Immungedächtnis in Invertebraten aktuell von Interesse, da es hilft, mehr über die Interaktionen von Wirt und Mikroorganismen zu erfahren. Das Immungedächtnis in Invertebraten erfüllt eine ähnliche Funktion wie in Vertebraten, beruht jedoch auf anderen molekularen Mechanismen.

Ich wollte die potentiellen Wege zur Erkennung von Mikroorganismen in Schwämmen untersuchen und entwickelte dazu einen experimentellen Ansatz. Der Schwamm *Halichondria panicea* konnte erfolgreich in einer Aquakultur gehalten werden. Im Experiment wurde er einem autochthonen *Vibrio* Stamm aus der Ostsee und einem exogenen *Vibrio* Stamm aus dem Mittelmeer ausgesetzt. Als Kontrolle diente steriles, künstliches Meerwasser. Die Immunreaktion wurde mit einem antimikrobiellen Assay und mit differentieller Genexpressions-Analyse (z.B., RT-qPCR des Zielgens *hsp70*) untersucht. Ich erwartete eine differenzierte Immunreaktion des Schwamms gegenüber den zwei unterschiedlichen Bakterienstämmen. Unter der Hypothese des Immungedächtnis, erwartete ich darüber hinaus eine stärkere Immunreaktion in *H. panicea* gegen den exogenen *Vibrio* Stamm im Vergleich zu dem autochthonen *Vibrio* Stamm. Die Schwämme wurden in einem Durchflusssystem mit der Suspensions-Methode nach Barthel & Theede (1986) gehalten. Die Probenentnahme fand nach 6h und 24h statt. Der antimikrobielle Assay zeigte die stärkste Immunreaktion nach 6 Stunden in Form eines größeren Halos. Insgesamt war die Reaktion gegenüber dem *Vibrio* Stamm aus dem Mittelmeer stärker. Die real-time quantitative PCR (RT-PCR) wurde für *actin* (Referenzgen) und *hsp70* (Zielgen) in *H. panicea* optimiert. Die Expression des heat shock protein Hsp70 war nach der Injektion mit dem *Vibrio* aus dem Mittelmeer erhöht. Diese Studie stellt neue Erkenntnisse zur Immunreaktion von Schwämmen, in diesem Fall *H. panicea*, gegenüber unterschiedlichen Bakterienstämmen dar und suggeriert Spezifität gegenüber unterschiedlichen Bakterien in diesen basalen Metazoen.

2. Abstract

Sponges (phylum Porifera) constantly encounter microbial cells, including potential pathogens, during their pumping activity, while harbour diverse and specific symbiotic microbial communities. However, how sponges detect and distinguish different microbes (e.g., symbionts *vs* food bacteria *vs* potential pathogens) remains unknown. I hypothesized that their innate immune system could be involved to provide specific recognition of microbes by ways of immune memory. I aimed to investigate potential pathways of bacteria recognition in sponges by adopting an experimental approach. First, an aquaculture flow-through system for Baltic sponges was optimized. Then, sponges were challenged with either an autochthonous *Vibrio* strain isolated from the Baltic Sea (VB) or an exogenous *Vibrio* strain isolated from the Mediterranean Sea (VM). Sterile artificial seawater was used as control. The immune response of the sponge was monitored by ways of antimicrobial assays and differential gene expression analysis (e.g., RT-qPCR of targeted gene *hsp70*). I expected a differentiated immune reaction of the sponge towards the two different bacteria strains. Moreover, under the hypothesis of immune memory, I expected a stronger immune response in *H. panicea* against the exogenous *Vibrio* compared to the autochthonous *Vibrio*. Sponges were successfully kept in a flow-through system with a suspension method according to Barthel & Theede (1986). Sampling occurred at two time points (6h and 24h). The antimicrobial assay showed the strongest immune reaction after 6 hours in form of a bigger halo diameter. The overall reaction was higher in the sponges treated with VM. The real-time quantitative PCR (RT-qPCR) was optimized for *actin* (reference gene) and *hsp70* (target gene) in *H. panicea*. The expression level of the heat shock protein Hsp70 was increased in the VM treatment. This study provides further insights in sponges' immune reaction to varying bacterial strains suggesting specificity towards different bacteria in these basal metazoans.

3. Introduction

Multicellular organisms arouse in a world dominated by microbes and, since then, animal evolution has been strongly influenced by animal-microbe-interactions (Nyholm & Graf 2012). Animals and microbes not only shared the same environment, but also get involved in stable symbiotic associations. The term "symbiosis", defined by Anton de Bary, is used to describe close interactions of organisms from different species, regardless of the benefits and costs derived from the association. Some microorganisms may be harmful (pathogenic) or beneficial (mutualistic) to the animal host, but in both cases they influence animal biology, ecology and development (Nyholm & Graf 2012). Symbiotic relationships, such as the Hawaiian bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* (Nyholm & McFall-Ngai 2004) or the symbiosis of corals and the dinoflagellate *Symbiodinium* sp. (Stat et al. 2008) are not just specialized exceptions. Every individual animal can be considered as a community of host and microbes (the so-called holobiont) and this new perspective has deeply impacted the understanding of the natural world (McFall-Ngai et al. 2013).

Animals require mechanisms to control and maintain homeostasis with symbiotic communities while preventing cheating or pathogenic infections (McFall-Ngai et al. 2013). Animal innate immunity can mediate specific microbial recognition and animal-microbe interactions (Nyholm & Graf 2012). Traditionally, the innate immune system has been considered as the mechanism for anti-pathogenic defense (Owen et al. 2009; Janeway & Medzhitov 2002). Most recently, evidence arouse that innate immunity is also involved in maintaining the equilibrium of symbiotic host-microbe interactions (Chu & Mazmanian 2013). The innate immune system can detect efficiently between self and non-self (Schulenburg et al. 2007) and is characterized by a quick response, within minutes to hours (Owen et al. 2009). The innate immune response to microorganisms relies on the recognition of molecules and molecular patterns associated with microbes. These molecular patterns were first termed PAMPs (Pathogen associated molecular pattern) but now are often named MAMPs (Microbe associated molecular pattern), as they are not restricted to pathogenic microorganisms. MAMPs are conserved, repeating components on the surfaces of microbes such as carbohydrate structures (peptidoglycan), lipopolysaccharides (LPS) or viral proteins. Pattern recognition receptors (PRRs) of animals recognize MAMPs and activate signal cascades, which lead to the expression of immune response proteins. The best known PRR is the Toll-like receptor (TLR) (Lemaitre et al. 1996) with extracellular leucine-rich repeats (LRRs) for

binding of MAMPs. Other PRRs are NLRs (NOD-like receptors), CLRs (C-type lectin receptors), RLRs (retinoic acid-inducible gene-I-like receptors) and SRCR (scavenger receptor cysteine-rich) (Owen et al. 2009; Mukhopadhyay & Gordon 2004; Hanington et al. 2010). Not all metazoan organisms contain the same receptor repertoires. Their structure may differ from the classical PRRs and functions of some receptors can also vary from the known function in vertebrates (degenerated TLR-pathway in Cnidaria: Hemmrich et al. 2007, Porifera: Srivastava et al. 2010; Riesgo et al. 2014; Bosch et al. 2009).

The TLR-signaling cascade seems to be highly conserved in animals and is one of the best described innate immunity pathways. After binding of a MAMP, the TLRs dimerize and the adaptor proteins, such as MyD88 (myeloid differentiation primary response 88 factor), attach to the intracellular TIR domain. This activates a signal cascade which involves several signal proteins, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF kB), where at the end yield changes in gene expression, such as synthesis of antimicrobial proteins. The activated effectors differ depending on the symbiont or pathogen encounter. For example, the pathway of antimicrobial peptides is known to build effective defensive weapons against pathogens (Zasloff 2002), whereas the cnidarian *Hydra* can express species-specific antimicrobial peptides to shape its commensal microbial community (Franzenburg et al. 2013). The innate immunity pathways are highly conserved and of early origin (Hemmrich et al. 2007) and the gene classes developed already before porifera and eumetazoa diverged (Larroux et al. 2006).

Invertebrate immunity can be highly specific, with different immune reaction upon different strains (Milutinovic & Kurtz 2016; Kurtz 2004). Specificity in the immune system seems to have primarily developed for adequate self/non-self recognition instead of pathogen defense (Kurtz 2004; Schulenburg et al. 2007). However, specific recognition of microbes would present evolutionary advantages not only to prevent the rejection of the symbiotic microbiota, but also to save the energetic investment of an inflammatory response against non-pathogenic microbes. Actually, mechanisms that combine specificity with immune memory would allow the organism to react faster and more effective on subsequent exposure to the same microbe by storing information about the first encounter. Immune memory was supposed to be restricted to vertebrates, but in recent years, evidences aroused that the concept also exists in invertebrates (ctenophores: Bolte et al. (2013); cnidarian: Brown & Rodriguez-Lanetty (2015)). Immune memory in invertebrates might be similar in function to vertebrate adaptive

immunity but based on different molecular mechanisms within innate immunity (Schulenburg 2007). The presence of immune memory in basal metazoan such as Ctenophora and Cnidaria (Bolte et al. 2013; Brown & Rodriguez-Lanetty 2015) suggests an ancient origin of this process and opens question on whether it is also present in other early diverged phyla, such as sponges.

Sponges (Phylum Porifera) belong to the phylogenetic oldest clades within metazoa and developed over 600 million years ago in the precambrian era (Li et al. 1998). Therefore, they are important for addressing evolutionary questions and identify conserved *vs* novel animal traits throughout animal evolution. Additionally, sponges represent a prominent example of complex animal-microbe interactions. When pumping water through their canal system, sponges encounter many different kinds of microbes, including potential pathogens and food bacteria, while harbor diverse and specific symbiotic microbial communities (Thomas et al. 2016; Taylor et al. 2007; Erwin et al. 2011; Webster & Taylor 2012). However, it is unknown how sponges detect and distinguish different kinds of microbes (e.g., symbionts vs food bacteria vs potential pathogens). Research on this early-diverging metazoan clade may provide insights into conserved mechanism of animal-microbe interaction (Pita et al. 2016; Thomas et al. 2016).

Sponges are abundant in all temperature zones including polar regions, from shallow water to the deep sea and also in freshwater ecosystems (Taylor et al. 2007). They play a significant role in benthic communities throughout the world. For instance, they influence nutrient cycles and ecosystem productivity by transferring dissolved organic matter to higher trophic levels, the so-called “sponge loop” (de Goeij et al. 2013). Additionally, they attract biotechnological interests for new pharmaceutical compounds produced by sponge and/ or its microbes (Mehbub et al. 2014; Indraningrat et al. 2016; Leal et al. 2012).

The simple poriferan body plan is unique among metazoans (Riesgo et al. 2014). Sponges do have epithelia, but lack true tissues and organs (Dunn et al. 2015). The pinacoderm separates the sponge from the surrounding seawater and builds the outer layer. Beneath the pinacoderm is the mesohyl, which is the functioning layer of the sponge (**Fig.1a**), and where metabolism, reproduction, nutrient transfer and cell communication components are located. Inside the mesohyl the totipotent amoeboid archeocytes and symbiotic microbes are distributed (Webster et al. 2007). Sponges are filter feeders and water enters the body via open pores in the

pinacoderm passing an aquiferous system of canals inside the sponge and exits the sponge via the osculum. The choanocytes are filtering cells and produce water flow with their continuous beating flagellum. Choanocytes are the cells, which are mainly exposed to the environment (**Fig.1b**).

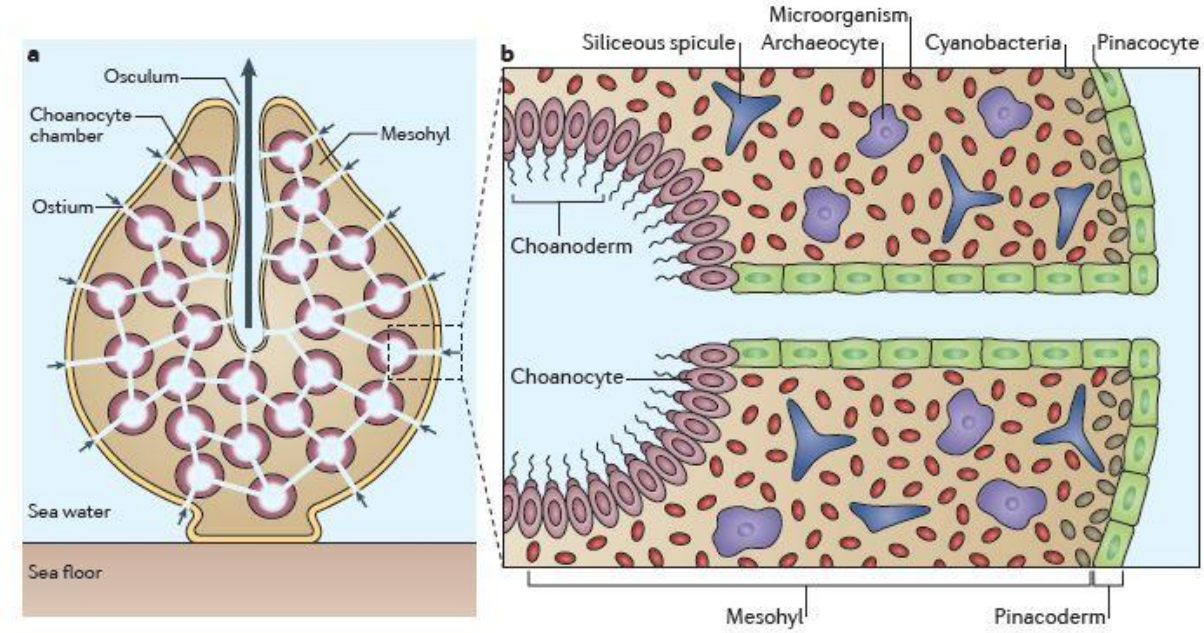

Fig. 1: **a** Scheme of typical demosponge bodyplan. **b** Section of the internal structures of a demosponge (original figures in Hentschel et al. 2012)

Despite the apparent simple body plan, genomic data suggests a high complexity in sponges (Riesgo, et al. 2014). Genomic information of several sponges, e.g. *Amphimedon queenslandica*, *Oscarella carmela*, *Stylissa carteri* and *Suberites domuncula* (Srivastava et al. 2010; Ryu et al. 2016; Riesgo et al. 2014) shows that sponges share an unexpectedly large complement of genes with other metazoans, including genes involved in cell–cell communication, signaling, or immunity. Several putative PRR-encoding genes were found in sponges, such as a TLR related receptor, LPS-binding-protein-like (LBL) proteins, putative NLRs and SRCR proteins. All of the PRRs present conserved domains but different structure to the classical PRRs described in other organisms, e.g. none of the sponge TLR-like receptors contains LRRs, what is normally the typical MAMP-binding site (Hentschel et al. 2012; Webster & Thomas 2016). Components of the immune signaling cascade, such as NF kB or MyD88, were found in sponge transcriptomes. However, the availability of innate immunity genes in the genome and transcriptome of sponges does not confirm the real function of the expressed molecule in the sponge (Riesgo et al. 2014). For instance, in *Caenorhabditis elegans* the TLR cascade is not involved in immunity (Couillault et al. 2004). In sponges, the function of MyD88

involved in the recognition cascade of gram-negative bacteria has been reported (Wiens et al. 2005), but the overall empirical evidence for gene functions in the innate immunity pathway is still scarce.

In this Master's thesis, I aimed to unravel the function of potential immune genes in sponges upon encounter with different bacterial strains. The low-microbial-abundance sponge *Halichondria panicea* of the Baltic Sea was exposed to heat-killed *Vibrio* bacteria and the immune response of the sponge was monitored by ways of antimicrobial assays and differential gene expression analysis (e.g., RT-qPCR of targeted genes). Heat-killed bacteria strains were successfully used in other invertebrate studies on immune challenge (Roth et al. 2009; Trapani et al. 2016; Zaragoza et al. 2014; Bolte et al. 2013) and *Vibrio* strains in general are commonly used for immune challenge experiments in marine invertebrates (Wright et al. 2013; Lokmer & Wegner 2015; Bolte et al. 2013). Two *Vibrio* strains were applied by injection of heat-killed bacteria in the mesohyl of the sponge. The sponge was supposed to be completely naive towards the exogenous *Vibrio* strain, whereas the autochthonous *Vibrio* strain might have been encountered before. I expect differential gene expression after *Vibrio* encounter and stronger response to the exogenous *Vibrio* strain, consistent with the evolutionary concept of reducing costs and self-damage of specific immune defense. By combining molecular analysis with an experimental approach, this study contributes to current research priorities in sponge microbiology, such as reveal host mechanisms involved in sponge-microbe interactions (Webster & Thomas 2016).

4. Material and Methods

4.1. *Halichondria panicea*

H. panicea (Pallas, 1766), with the common name "breadcrumb sponge" is a marine sponge of the class Demospongiae. It belongs to the family *Halichondriidae* and the genus *Halichondria*, with 109 accepted species and more than 200 unconfirmed species (WoRMS, 28.01.2017). *H. panicea* is widely distributed in the Northern Hemisphere from the Baltic and North Sea to the North Eastern Atlantic, with sister groups in the North Pacific (Erpenbeck et al. 2004). The main habitat is the intertidal zone, but *H. panicea* can also appear in the sublittoral and down to 500 m depth. Several morphotypes are known for *H. panicea*: compact, encrusting and branched forms. Also, their color varies between yellow, grey and greenish.

The specimens investigated in this study belonged to the Baltic Sea population of *H. panicea* from Kiel Bight, where this species is one of the most abundant species of sponges (Barthel 1986). They live mainly on Red algae of the phylum *Phyllophora* sp. or *Phycodrys* sp., but also on hard substrates such as rocks. Growth, reproduction, spermatogenesis, energy budget and biomass production were extensively investigated (Barthel & Detmer 1990; Witte et al. 1994; Barthel 1986). Their lifecycle starts in spring from a planktonic larval stage that transform to the adult sponge after settling on either hard substrate or red algae. Over the summer period the sponge' body volume increases until it reaches the maximum in August. With progressing seasons the sponge mass decreases and almost disappears in winter (Barthel, 1985; 1986; 1988). Every few weeks *H. panicea* sloughs off its outer tissue. The cause is unknown, but is hypothesized that could be a mechanism to prevent sedimentary clogging of its ostia or fouling (Barthel & Wolfrath 1989). Despite the intensive studies on physiology, morphology and ecology, only little genetic information is available on *H. panicea* and its genome is not sequenced.

Because of their high abundance *H. panicea* plays an important role in the habitat of the Kieler Bay e.g. by providing nutrients to the surrounding seawater (Barthel 1988). The genus *Halichondria* is also relevant for biotechnological interests for the production of antimicrobial, antifungal or cytostatic compounds, either produced by the sponge itself or by associated microbes (Blunt et al. 2007; Clark et al. 1992). Finally, due to its amenability to aquaculture, it has become a potential model organism for the study of host-microbe-environment

interactions at Geomar (Pita et al. 2016). The morphotypes of *H. panicea* in this study were yellow and red colored and of branched form.

4.2. Sponge aquaculture

Sponges of the species *H. panicea* were provided by Claas Hiebenthal (KIMOCC, Geomar Helmholtz Centre for Ocean Research Kiel) and sampled at Kieler Mussel farm (54° 22.558'N, 10° 9.786'E), Baltic Sea at 6 m depth. Cultivation conditions were orientated on the work of Barthel & Theede (1986) and Westphal (1988). The sponges were maintained in an open flow-through system with direct uptake of Baltic Sea water, which will keep biological and physical parameters of the water at similar conditions (temperature; pH; salinity) as in the field. The water is provided via a header tank to oxygenate the incoming water to the aquaria (**Fig. 2**).

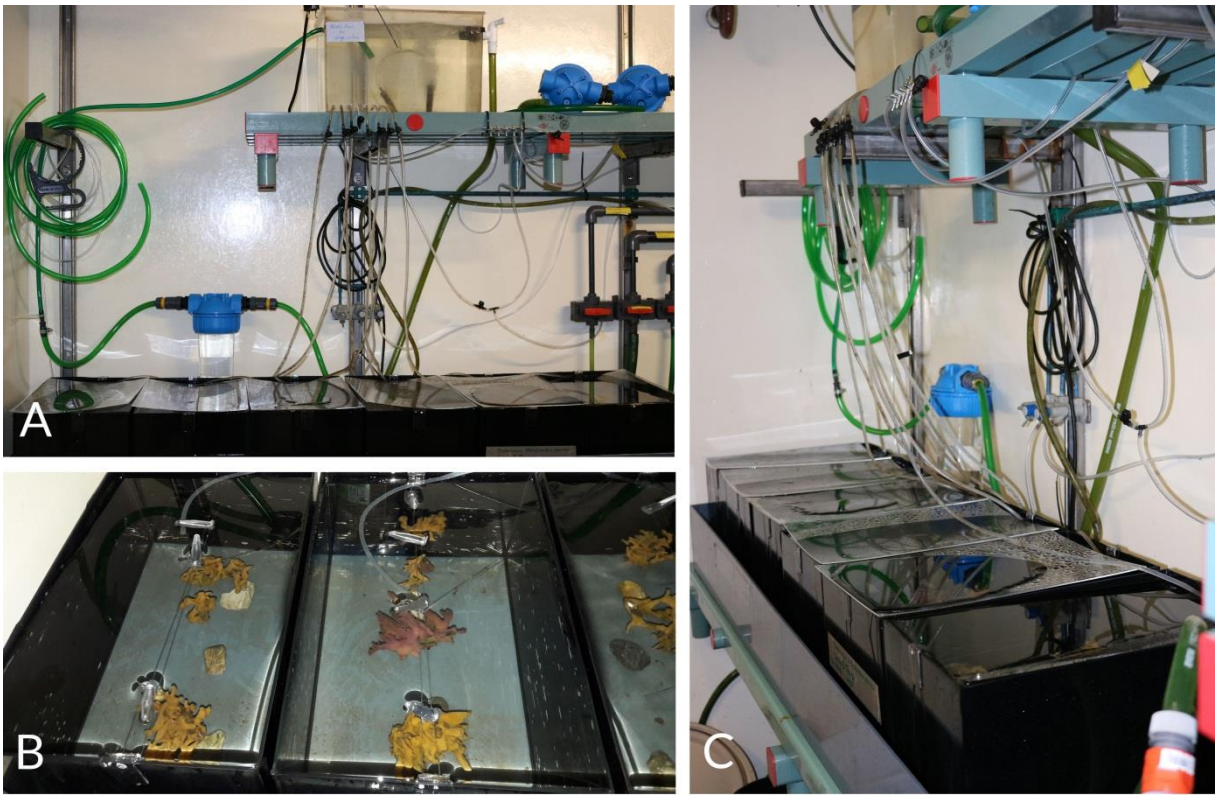

Fig.2: A, C Sponge aquaculture with a flow-through system. **B** sponge individuals in the tanks.

H. panicea lives regularly attached to algae and therefore a floating state enhance its maintenance in aquaculture (Barthel & Theede 1986). Sponges were attached to nylon strings on top of the aquarium and to a small stone on the bottom (as weight) with a 0.2 mm nylon string (**Fig. 3**). PE beads were added under the sponges to prevent them from sliding down. Handling of sponges occurred always under water to avoid air embolization.

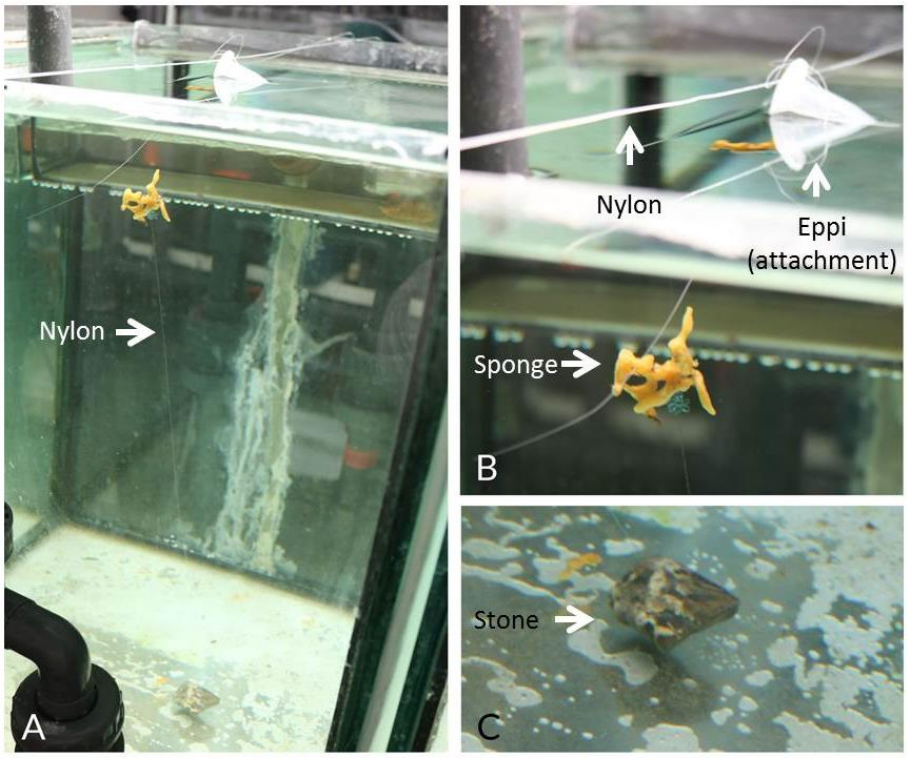

Fig.3: Attachment of sponges (here in Experimental aquarium system).

Each aquarium was 25x40x25 cm big and covered outside by black foil to simulate the low light conditions in the red algae zone. The top of the aquaria were covered with Plexiglas lids. A filter system of three filter stages (50µm, 10µm, 5µm) was tested to reduce sediment intake in aquaria. However, it had to be removed due to quick cloaking of filters (i.e., within hours), which could stop the water flow in the system.

After setting up the system, a cleaning and care protocol was developed and included weekly cleaning, feeding and water physical parameter measurement. Sponges were fed with *Chlorella* algae powder (2 mg per sponge once per week, as estimated according to Barthel & Theede 1986). Physical parameters were monitored by HOBBO data loggers (temperature and light; continuous measure) and a multisensory (salinity, pH, oxygen; twice per week). Moreover, water samples for flow cytometry were collected to estimate the concentration of bacterioplankton in the seawater at aquarium facilities and direct from the Kiel Bight outside of Geomar.

Sponge health was assessed by visual inspection and monitoring of sponge pumping activity. Pumping activity was tested via the use of fluorescein dye before the experiments. The Fluorescein dye was solved in sterile artificial seawater (15 psu) and added to the water close to the osculum of the sponge. The water stream leaving the osculum was moving the dye front, what indicated active pumping and therefore physiological activity of the sponge (**Fig. 4**).

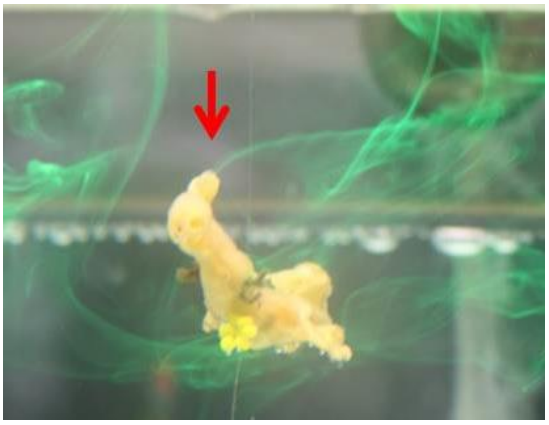

Fig. 4: Fluorescein dye makes sponge pumping activity visible. Red arrow: Dye exits osculum.

Six sponges were sampled right after arrival to the Institute, within hours after collection (= wildtype condition) for phylogenetic analysis and optimization of protocols. Sampling was performed with sterile knives. Samples for gene expression analysis were fixed in RNAlater and stored at -80°C until they were processed. Samples for antimicrobial assays were directly frozen and stored at -80°C.

4.3. DNA and RNA extraction

RNA and DNA of the six wildtype sponges were extracted with the AllPrep DNA/RNA MiniKit (Qiagen). The protocol was optimized for *Halichondria* (tissue amount, times) (**appendix 9.3.1**). RNA extracts were treated with an Anti-RNase to protect the RNA from degrading and DNA-nuclease to remove possible DNA contamination. DNA contamination of RNA samples was excluded via PCR with Euk18S primer and agarose gels. Quality of the treatment was quantified with Experion chip (**appendix 9.3.3**). RNA concentration and quality was assessed with Nanodrop and Qubit. RNA extracts were stored at -80°C. DNA extractions were used for phylogenetic analysis, while RNA samples were used for optimization of RT-qPCR.

4.4. Phylogenetic analysis

A molecular phylogenetic analysis was performed on the two morphotypes of *H. panicea*. The phylogenetic analysis was performed in the six wildtype samples using four different markers: COI mtDNA, 18S rRNA, 28S rRNA and ITS-2 genes. The primers used for PCR and sequencing are presented in **Table 1**. After PCR amplification, cleaned-up PCR products were sent for sequencing. Quality of sequences was estimated with Chromas software and primer sequences were removed. Sequences were blasted in NCBI.

Phylogenetic trees were designed based on COI mtDNA, 18S rRNA and ITS-2. The phylogenetic trees included the sequences that were generated in this study, other porifera from the NCBI database, cnidarian, ctenophores and *Mus musculus*, respectively *Stichopus monotuberculatus* as outgroup. The sequences were aligned with MAFFT or MUSCLE and reduced to the same length. A maximum likelihood tree was prepared in MEGA 6 with 1000 bootstrap.

Table 1: Phylogenetic markers and primers applied to PCR amplification and sequencing.

Gene	Primer	Primer sequence	Reference
18S rRNA	SP18aF	5'-CCTGCCAGTAGTCATATGCTT-3	Redmond et al. 2013
	SP18gR	5'-CCTTGTTACGACTTTTACTTCCTC -3'	Redmond et al. 2013
ITS-2	SP58bF	5'-AATCATCGAGTCTTTGAACG-3'	Thacker & Starnes 2003
	SP28cR	5'-CTTTTCACCTTTCCCTCA-3'	Thacker & Starnes 2003
COI mtDNA	dgLCO	5'-GGTCAACAAATCATAAAGATATTGAYATYGG-3'	Meyer et al. 2005
	COX1-R1	5'-TGTTGRGGGAAAAARGTTAAATT-3'	Rot et al. 2006
28S rRNA	Euk28S_26F	5'-ACCCGCYGAAYTTAAGCATA-3'	Stewart et al. 2010
	Euk28S_3126R_T7	5'-AATTATAATACGACTCACTATAGATTCTGRYTTAGAGGCGTTCAG-3'	Stewart et al. 2010

4.5. Experimental set-up for immune response experiment

Three treatments were applied for the immune response experiment by direct injection into the sponge mesohyl (50 µL): control (sterile filtered artificial seawater 15 psu), heat-killed *Vibrio* from the Mediterranean (VM) and heat-killed *Vibrio* from the Baltic Sea (VB). *Vibrio* spp. are abundant in the bacterioplankton communities of coastal waters and some species are pathogens of marine animals, including invertebrates such as sponges, cnidarians and corals (Thompson et al., 2004; Roth et al., 2012). These gram-negative bacteria were already used successfully for other immune challenging studies (Zaragoza et al. 2014; Trapani et al. 2016; Bolte et al. 2013; Roth et al. 2009). The *Vibrio* from abroad is from the Mediterranean Sea in Italy kindly provided by Olivia Roth (FB3 EV, Geomar Helmholtz Centre for Ocean Research Kiel). The *Vibrio* from the Baltic Sea is *Vibrio* sp. PP-XX7 sampled 2010 in Strande/Baltic

Sea from muddy ground in 5 m depth. It was kindly provided by Jutta Wiese and Tanja Rahn (FB3 MI, Geomar Helmholtz Centre for Ocean Research Kiel).

Vibrio strain cultures were reactivated according to Bolte *et al.*, 2013. *Vibrio* phylotypes were taken from a frozen glycerol stock (40% glycerol) and grown in medium 101 (5 g Peptone and 3 g meat extract per liter) adjusted for marine bacteria by addition of 1.5% NaCl and incubated at 25°C at 180 rpm overnight. Bacteria cultures were transferred into 1.5 ml Eppendorf tubes, heat deactivated at 65°C for 1h, centrifuged at medium speed (2000 rpm) and then the bacterial pellet was resuspended in artificial seawater (AquaMedic, 15psu, sterile filtered 0.22 µm).

The experiment took place in a flow-through system of 18 aquaria kindly provided by Olivia Roth (FB3 EV, Geomar Helmholtz Centre for Ocean Research Kiel). Water samples were taken from the aquaria before the experiments started, on both experimental days and directly from the Kiel Bight before and after the experiments to analyze bacterioplankton concentration in the seawater by flow cytometry. Samples were fixed with Paraformaldehyde and Glutaraldehyde (**appendix 9.3.3**) to a final concentration of 1% and stored directly at -80°C. Flow cytometry was performed at the flow cytometer FACScalibur (Becton & Dickinson) of FB3 Research Unit "Marine Food Webs", access kindly provided by Thomas Hansen. Samples were diluted (1/4) with artificial seawater (16 PSU). Heterotrophic bacteria in the water were stained with SYBRGreen solution (final concentration of 0.025% (1:20.000)). The amount of bacteria was measured at flow rate 12 µL/min, threshold 2 min and green fluorescence (FL1). Bacterial cells were recorded in log-scale and were identified according to their size and fluorescence (settings: FL1 vs. SSC; FL1 threshold: 144, FSC: E02, SSC: 460). Data analysis was performed with Microsoft® Excel and FlowingSoftware 2.5.1.

For the first immune response experiment (IR), three sponges were divided in three clones and each clone was kept in an individual aquarium and assigned to one treatment. Thus, three replicates per treatment. They were kept in the aquaria for 4 weeks for acclimation. The clones of one sponge died a few days before the experiment started. Therefore, the replicates were reduced to two per treatment. No food was provided during the experiment. In addition the first immune response experiment (IR) was performed simultaneously in three *Haliclona* sp. with the conditions described above and a second immune response experiment (IR2) was performed with three replicates of *H. panicea* three months after the first experiment.

Sponge material was sampled 6h and 24h after the injection from two different areas (close and distant to the injection zone) to estimate the time of strongest immune response and investigate local or generalized immune response (**Table 2**). Sampling was performed with sterile knives. Sponge samples for gene expression analysis were fixed in RNAlater and stored at -80°C until they were processed. Sponge samples for antimicrobial assays were directly frozen and stored at -80°C.

Table 2: Experimental design

Sampling time point:	**6h**	**24h**
Challenge:		
Sterile artificial seawater	S (control) 6h	S (control) 24h
***Vibrio* from Baltic Sea**	VB 6h	VB 24h
***Vibrio* from Mediterranean Sea**	VM 6h	VM 24h

4.6. Antimicrobial assay (AM)

Sponges' immune reaction of the first immune response experiment (IR) was analyzed via an antimicrobial assay. The assay took place in the S2 laboratory of the FB3 EV group. The protocol of (Roth *et al.*, 2012) was optimized for the sponge material. For implementing the protocol, two wildtype sponges without treatment were tested. Overnight culture of bacteria was transferred to a larger volume and kept in exponential growth for additional 2h. Then, optical density (OD) was measured at 600nm in a Spectrophotometer (Nanodrop). *Vibrio* from the Mediterranean was grown to an optical density of 0.032 and *Vibrio* from the Baltic Sea was grown until OD was 0.016. Then, 5mL of medium 101 overlay agar were infused with either 1mL of *Vibrio* from Italy or 1mL of *Vibrio* from the Baltic Sea and poured on plates. The optimization showed no difference between fresh and frozen sponge tissue, so frozen samples from the experiment were taken. Sponge pieces were placed on medium 101 overlay agar plates. Tetrazycline (1 mg/mL) was used as positive control. MilliQ water was used as a negative control. The experiment was performed in triplets with two pieces of the tissue on each plate, resulting in six technical replicates for each sponge clone and condition (**Fig. 5**).

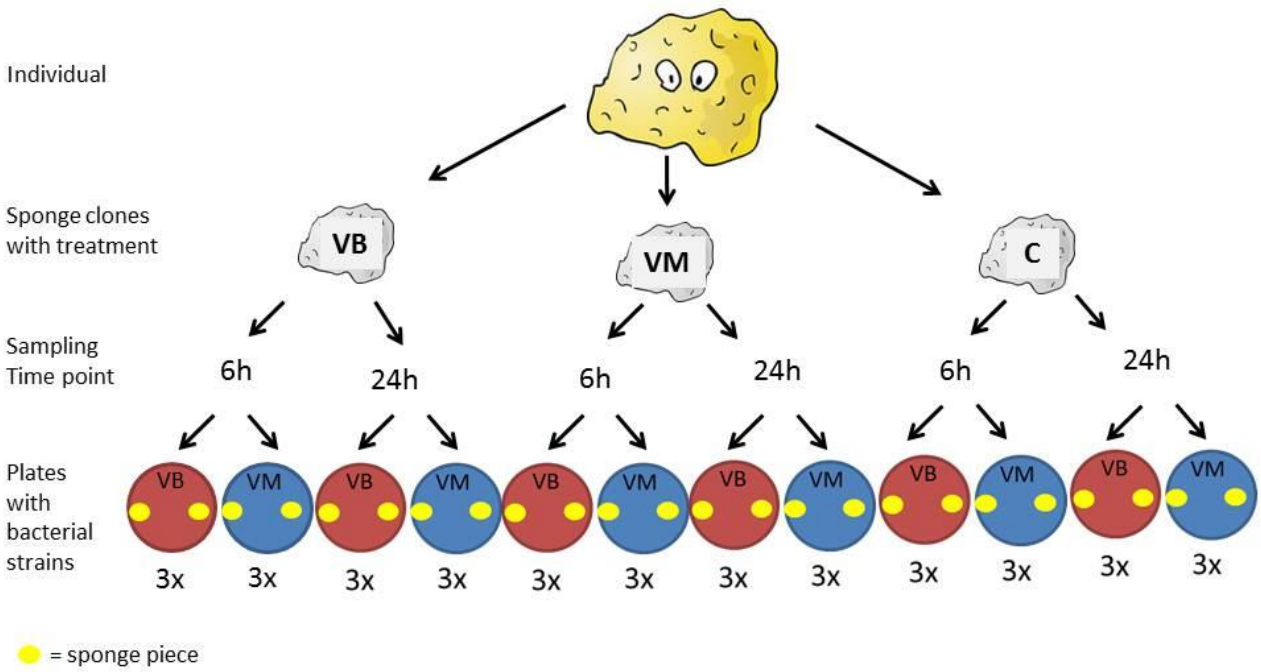

Fig. 5: The procedure of the antimicrobial assay. The sponge was cut in clones and treatments were applied according to the IR experiment (see section Immune response experiment). Samples were taken on two time points. Each time point sample was places in duplicates on two different bacterial infused plates (VB & VM). All plates were prepared in triplets.

Plates were incubated at 25°C for 16–20h. Diameters of inhibition zones were measured to the nearest 0.1 mm. Plates were photographed with a DSLR camera. Data analysis was performed with RStudio. Halo size was estimated by subtracting the sponge piece diameter from the inhibition zone diameter. For statistical analysis Shapiro-Wilks-test for normality and Bartlett-test for equality of variances were applied. Significant differences were tested with Kruskal-Wallis-test and ANOVA (only complete and *Haliclona* dataset, not *Halichondria* dataset, because only two replicates were finally available). Both statistical test were applied because the Kruskal-Wallis-test is robust against non-normal distribution of data, but cannot consider individual error (e.g. it counts every replicate as individual), whereas the ANOVA considers the individual error but is less robust against non-normal distribution.

4.7. Candidate genes and primer design for RT-qPCR

The candidate genes for investigation of the immune reaction and the reference genes were chosen after study of recent literature (Boehm *et al.*, 2000; Boehm *et al.*, 2001; Wiens *et al.*, 2005; Bolte *et al.*, 2013; Redmond *et al.*, 2013; Milutinovic *et al.*, 2016; Rodrigueez-Lanetty et al. 2008) (**Table 3**). The cytoskeletal structure protein Actin and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), involved in glycolysis are often used as reference gene

in RT-qPCR analysis, also in studies in invertebrates (Bolte et al. 2013). Also, some authors have used 18S rRNA gene as reference gene (Li et al. 2014) . Members of the heat shock protein family are highly conserved and function as molecular chaperones by protecting the organism against thermal or other stress-induced damage (Borchiellini et al. 1998). They are also involved in intracellular protein transport and protein biogenesis (Shimpi et al. 2016) and in immune challenge response (Brown & Rodriguez-Lanetty 2015; Brown et al. 2013). MyD88, JNK and p38 are interesting as components of the TLR signaling pathway and phenoloxidase and peroxiredoxin as effectors.

As no genome information is available for *H. panicea*, the primers were designed by using aligned sequences of other sponge species found in NCBI and focused on the most conserved areas. Sequences were aligned by MUSCLE in MEGA6. Degenerated primers were designed with IDT PrimerQuestTool for primer design and evaluated with IDT OligoAnalyzerTool and the TM calculator of Thermofisher. The designed primers were tested on cDNA of the six wildtype samples via touch down PCR and evaluated via agarose gels. After appearance of clear single bands, the PCR product was cleaned up with the DNA, RNA and protein purification Kit (Macherey and Nagel) and send to sequencing. Sequences were analyzed with Chromas for quality of sequence chromatogram and sequences were edited (e.g., removal of primer sequence) in BioEdit v7.2.5. Sequences were blasted against the NCBI database. From the long sequences of the investigated genes, shorter fragments (75-200bp) were designed, which were required for qPCR analysis. Optimal annealing temperature was estimated with gradient PCR. Fragments were sequenced again to guarantee the right target.

Table 3: Candidate genes (*= optimized genes)

Target genes	**expression pattern**	**reference**
HSP 70*	General stress indicator	Brown et al. 2013
MyD88	Involved in TLR signalling pathway, Significant increase with LPS exposure	Wiens et al. 2005
Phenoloxidase	Effector, significant lower in the homologous treatment, involved in immune priming in beetles	Bolte et al. 2013, Milutinovic et al. 2016

Peroxiredoxin	Effector, Antioxidant, induced by LPS	Bolte et al. 2013
JNK protein kinase	Involved in TLR signalling pathway, activated by LPS exposure	Boehm et al. 2001
Protein kinase p38	Involved in TLR signalling pathway, activated by LPS exposure	Boehm et al. 2000
Reference genes		
ß-actin*	Reference gene	Rodriguez-Lanetty et al. 2008
GADPH	Reference gene	Bolte et al. 2013
18sRNA	Reference gene	Redmond et al. 2013

4.8. RT-qPCR optimization

As the results of the antimicrobial assay suggested higher immune reaction after 6h, I chose that time point for the gene expression analysis. RNA and DNA extractions of immune response experiment samples were performed for the 6h time point following the optimized protocol described above. One RNA sample of each experimental clone was reverse-transcribed into cDNA (**appendix 9.3.2**) and used as template for real-time quantitative PCR (RT-qPCR). Candidate and reference genes were optimized and RT-qPCR design was established to test gene expression level of the samples from the different treatments in *H. panicea*. Primers for myeloid differentiation factor 8 (MyD88), involved in the signaling cascade of PRRs, and the reference gene GAPDH were designed for *Haliclona* sp., but the RT-qPCR assay could not be optimized yet.

RT-qPCR detects and measures the increase or decrease of expressed genes under different conditions. The detection of PCR products is provided by including a fluorescent molecule (her: SYBRgreen) that stains double-stranded DNA. The increase of double-stranded DNA amount is proportional with the increase of the fluorescent signal. Based on a set-up with a target gene (changes expression level under treatment) and a reference gene (does not change expression level under treatment) the results of the treatment can be evaluated. Verification of

absolute values and relative quantification is possible. In this study relative quantification was performed.

Important requirements for a comparable set-up are reliable reference genes and primers with similar amplification efficiencies (to guarantee comparability between target and reference genes). For an estimation of primer efficiency a standard curve of DNA samples of known (for absolute quantification) or unknown (for relative quantification) concentration can be performed. The standard curve should be performed in doubles. The replicate reactions should be consistent and the standard curve should be as linear as possible (R^2>0.98). The amplification efficiency of the primers should be high (90–105%) and within a range of 5% to each other to perform convenient evaluation methods such as, $2^{-\Delta\Delta Ct}$ (Livak) Method or the ΔCt Method using a Reference Gene. In this study the primer efficiencies differed from each other (see results). Therefore, the Pfaffl-Method was used.

Formula Pfaffl-Method

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T,\ \text{target (calibrator – test)}}}{(E_{\text{ref}})^{\Delta C_T,\ \text{ref (calibrator – test)}}}$$

The above equation assumes that each gene (target and reference) has the same amplification efficiency in test samples and calibrator samples, but it is not necessary that the target and reference genes have the same amplification efficiency as each other (BIO-RAD Laboratories 2006).

The standard curve was performed from a dilution series of wildtype cDNA with 1/5 dilution. The dilution was prepared in tRNA water (10ng/µl). tRNA is a small oligonucleotide that does not disturb the reaction but keeps the template in solution by flattening uneven tube walls. This improves the homogenic solution of cDNA template in the tube.
The standard curve was performed in doubles for each gene with wildtypes. Each well of the 96-well plate contained SYBR-Green qPCR buffer (1x), forward and reverse primer (300 nml), 5µl template (cDNA) and molecular H_2O. Protocols for RT-qPCR conditions were optimized for each gene. Reagents were ordered at ThermoFisher®Scientific. H_2O was used as negative control.

4.9. RT-qPCR experiments

Differential gene expression analysis of *hsp70* and *actin* was performed for *H. panicea*.
Two qPCR experiments (= two plates) were performed with reagents described above. The first plate contained *H. panicea* sponge samples from the first immune response experiment (IR), which were two biological replicates (clones) per treatment. The second plate contained *H. panicea* sponge samples from the second immune response experiment (IR2), which were three biological replicates (no clones) per treatment. Three technical replicates per treatment were placed on the plate. Target gene was *hsp70* and reference gene was *actin*. Analysis of Ct ratio was performed with RStudio and Microsoft®Excel 2010 using the Pfaffl-Method. In IR2 the ΔCt of the calibrator (=control sample) was the mean of either HSP70 controls or actin controls to provide more homogeneity to the data, because the biological replicates were not clones in the IR2. For statistical analysis Shapiro-Wilks-test for normality, Bartlett-test for equality of variances and t.test (only complete and IR2 dataset, not IR dataset) were applied.

5. Results

5.1. Phylogenetic analysis

For my study, I received two different morphotypes of *H. panicea* in the Kiel Bight (**Fig. 6**). One morphotype was yellow colored with a harder and more brittle tissue. Sponges of this morphotype grew very branchy and attached to the nylon. Most of them were associated with small tubeworms in brown fragile tubes. After three weeks in the aquarium, some of the yellow individuals started to drop pieces, which attached to the glass bottom of the aquarium. The other morphotype was rose or red with a soft and elastic tissue. The individuals grew also branchy, but appeared to be more compact than the yellow ones. Throughout the experiment they were more covered with sediment than the yellow individuals. The phylogenetic relationship between the sponges was analyzed for three individuals of each morphotype based on four molecular markers: the COI mtDNA gene, the ribosomal 18S rRNA and 28s rRNA gene and the second internal transcribed spacer (ITS-2).

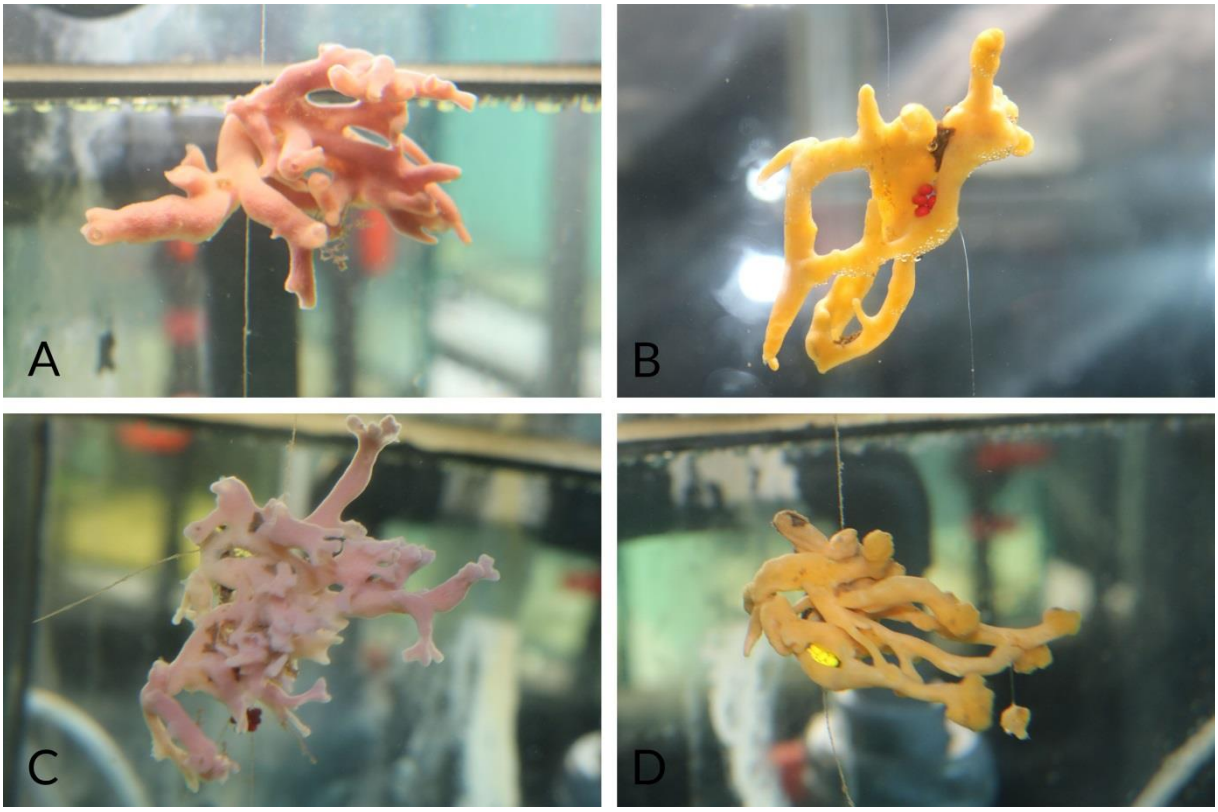

Fig. 6: A & C show the red morphotype of the sponge, B & D show the yellow morphotype of the sponge

The sequences (983-1200 bp long) of the COI mtDNA primers identified the yellow morphotype with 99% identity as *Halichondria panicea* (KC869423.1). The yellow morphotypes were 99.58-99.83% identical. The red morphotype sequences were 99.15-99.66% identical and showed 98%-99% identity with *Haliclona sp*. (JN242210.1). In the phylogenetic tree based on COI mtDNA sequences the yellow morphotype clearly (bootstrap 100%) clusters together with *H. panicea* (KC869423.1). The red morphotype clusters together with *Petrosia sp*. (JN242220.1) (bootstrap 43%) in short distance to *Haliclona sp*. (LC126249.1). The two morphotypes clearly cluster separately (**Fig.7**).

The sequences of 18S rRNA gene (793bp - 1169bp long) of the yellow morphotype showed 98%-99% identity with *Halichondria panicea* (KF699110.1). The 18s RNA gene sequences of the red morphotype showed 87% - 95% identity with *Oceanapia* sp. (DQ927317.1) and 90%-95% identity with *Haliclona sp*. (EU095523.1). All three yellow morphotypes are 99% identical and red morphotype sequences were 94-98% identical. Identity between yellow morphotypes and red morphotypes was 84-90%. The ML tree shows clearly that the two morphotypes do not cluster together (**Fig.8**). The yellow morphotype clusters together with *H. panicea* sequences (KF699110.1) (bootstrap 98%), whereas the red morphotype clusters separated (bootstrap 91%) next to *Oceanapia* sp. (DQ927317.1), *Xestospongia muta* (AY621510.1) and *Haliclona* sp. (AY734444.1). Ctenophore sequences cluster within the sponge sequences. In contrast, the marker ITS-2 (520bp - 580bp long) revealed that all six wildtypes were closely-related to *Halichondria sp.* (AF062607.1) (99%-100% identity, AF062607.1) and clustered together with *Halichondria panicea* (AF062607.1) in the ML phylogenetic tree (**Fig.9**). 28S rRNA sequences (1049 - 1234 bp long) were only successfully amplified for the yellow morphotype. The sequences showed 98-99% identity with *H. panicea* (AF062607.1, HQ379242.1).

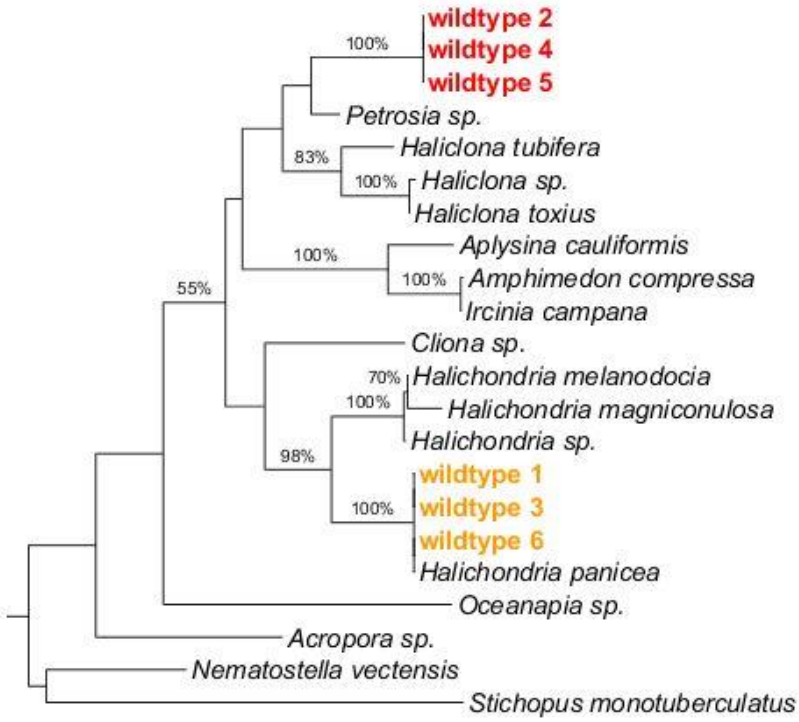

Fig. 7: Maximum likelihood tree based on COI mtDNA sequences (Genebank ID **appendix 9.2.5**).

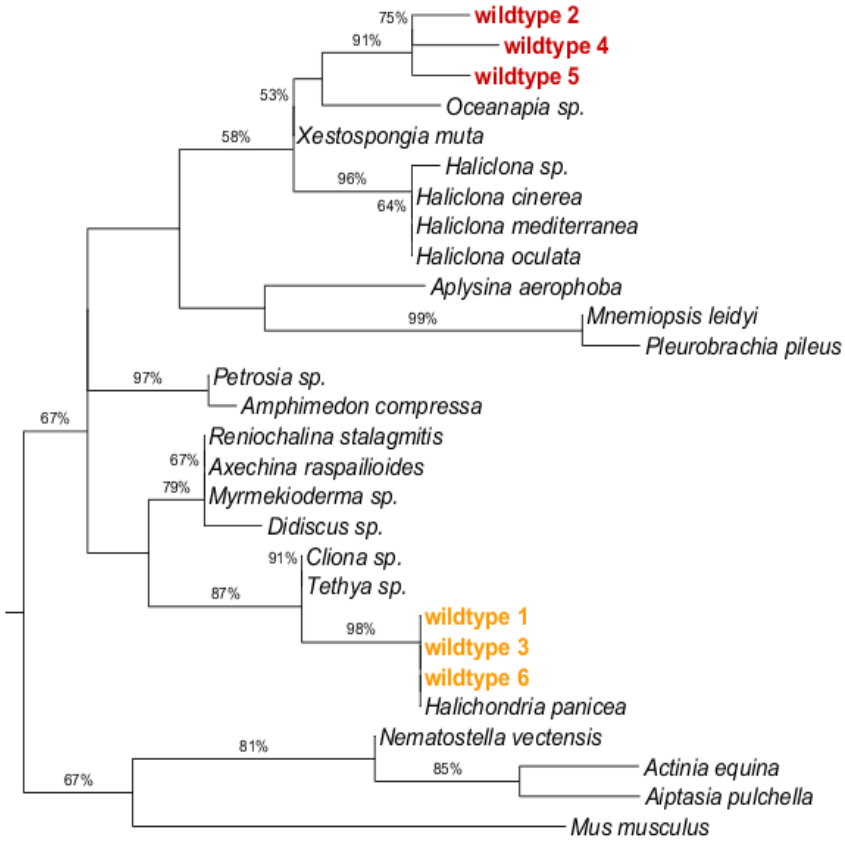

Fig. 8: Maximum Likelihood phylogenetic Tree based on 18S rRNA gene sequences (Genbank ID **Appendix 9.2.5**).

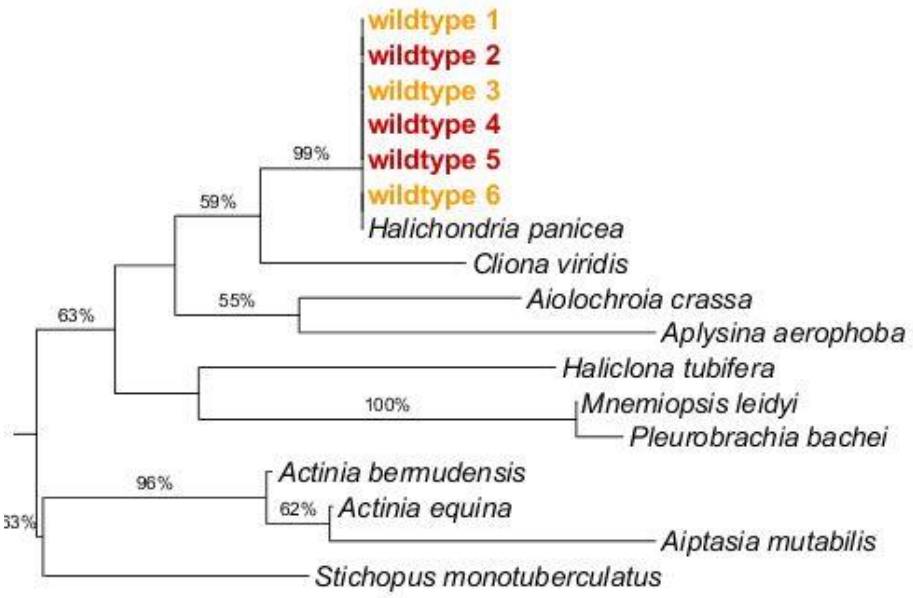

Fig. 9: Maximum Likelihood phylogenetic Tree based on ITS-2 gene sequences (Genbank ID **Appendix 9.2.5**).

5.2. Sponge aquaculture

Both sponge species were kept for several months in the flow-through system. *Haliclona* sp. sponges were kept for 12 weeks before the color changed to a pale red. At this state, no pumping activity could be detected anymore and the sponge material finally dissolved in the water. *H. panicea* sponges were kept for 21 weeks in the aquaculture system. During the whole aquaculture period, *H. panice*a sponges were shrinking in size and dropping pieces. These pieces actively attached to the glass bottom of the aquarium and started growing again. Over the whole aquaculture period, two events occurred were four sponges of *H. panicea* started molding. Two of them grew in the aquaculture tanks and two of them grew in the experimental tanks (**Fig. 10**). The incidence occurred over night. The sponge body was covered in a grey biofilm and the inner sponge material was dark grey or black and smelled like rotten eggs. No molding was observed for *Haliclona* sp.

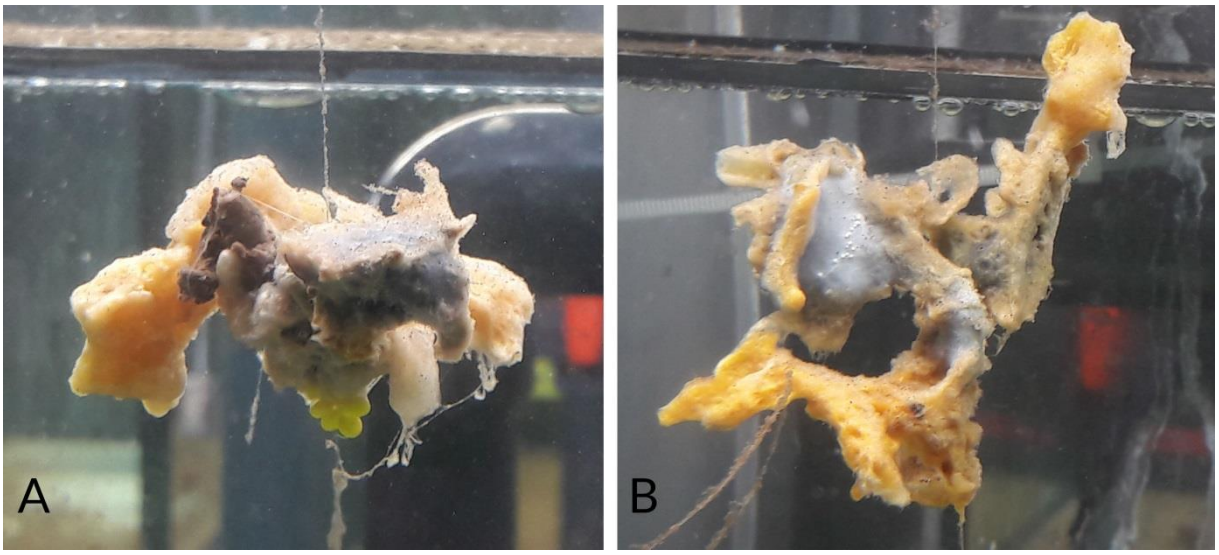

Fig 10: A and **B** show molding *H. panicea* sponges in the experimental tanks.

5.3. Immune response experiment

The conditions of water temperature, salinity, pH, oxygen in water and light were controlled throughout the whole experimental period of the immune response experiments. First experiment (IR) was performed in November 2016. The temperature was constant at 13°C. After this first experiment, the experimental system was kept running and physical parameters were monitored weekly. Water temperature decreased according to seasonality over the aquaculture period from 13°C in November 2016 to 11°C in February 2017, when the second experiment (IR2) was performed. All other conditions remained constant (**appendix 9.2.2, 9.2.3**).

During the first experiment, the bacterial load was checked and compared to the bacterial load of the water in Kiel Bight (**Fig. 11**). The overall bacterial load stayed constant throughout the three days of experiment, ranging between 1.96 x 10^6 to 1.60 x 10^6 bacteria per mL seawater. Values around 2x 10^6 bacteria per mL seawater are normal for seawater from Kiel Bight (Rheinheimer 1996, pers. comm. Thomas Hansen; pers. comm. Carlo Berg). The bacterial composition differed slightly between Kiel Bight water (**Fig. 11 probe 1 & 2**) and aquarium water (**Fig. 11 probe3-6**).

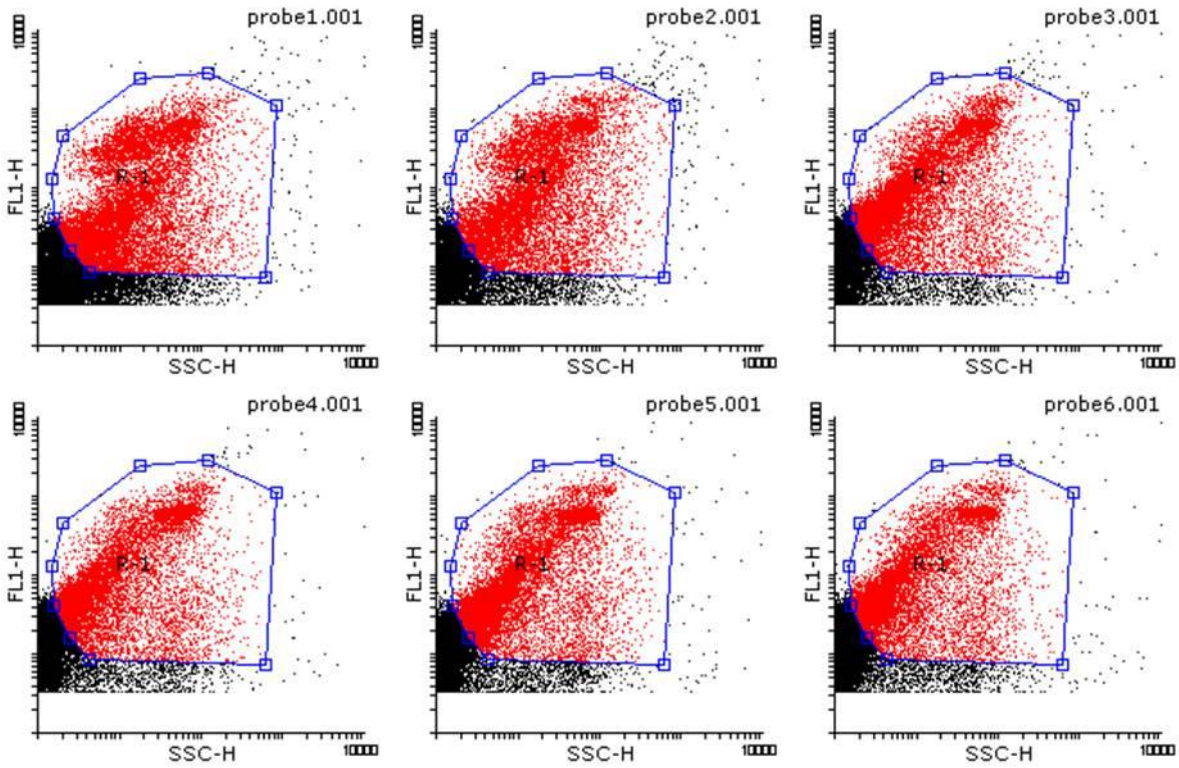

Fig. 11: Flow cytometric analysis of surface seawater in Kiel Bight (probe 1-2) and in the experimental aquaria (probe 3-6) after staining with SybrGreen I. Acquired events are displayed in log mode. Each graphs represents green fluorescence (FL1-H) vs 90° light scatter (SSC-H). The gate shows the events that have been counted. Different bacterial subpopulations are distinguished.

5.4. Antimicrobial assay

The antimicrobial assay estimated the time point of strongest antimicrobial reaction for later correlation with and indications for bacteria-specific antimicrobial activity. The two *Vibrio* strains (from Baltic Sea and from Mediterranean Sea) grew in different densities on the 101-medium agar plates. *Vibrio* from the Baltic (VB) grew less dense than *Vibrio* from the Mediterranean (VM). The antimicrobial assay was performed against VB and VM plates for each experimental sample (control, exposed to VB, exposed to VM) collected at two time points (6h, 24h). On plates with *Vibrio* from the Mediterranean, all sponge samples including control showed an antibacterial reaction with clear halos around the sponge pieces. On plates with *Vibrio* from the Baltic, all sponges showed a biostatic reaction were bacteria were still visible in the halos but in lower density than the surrounding medium (**Fig. 12**).

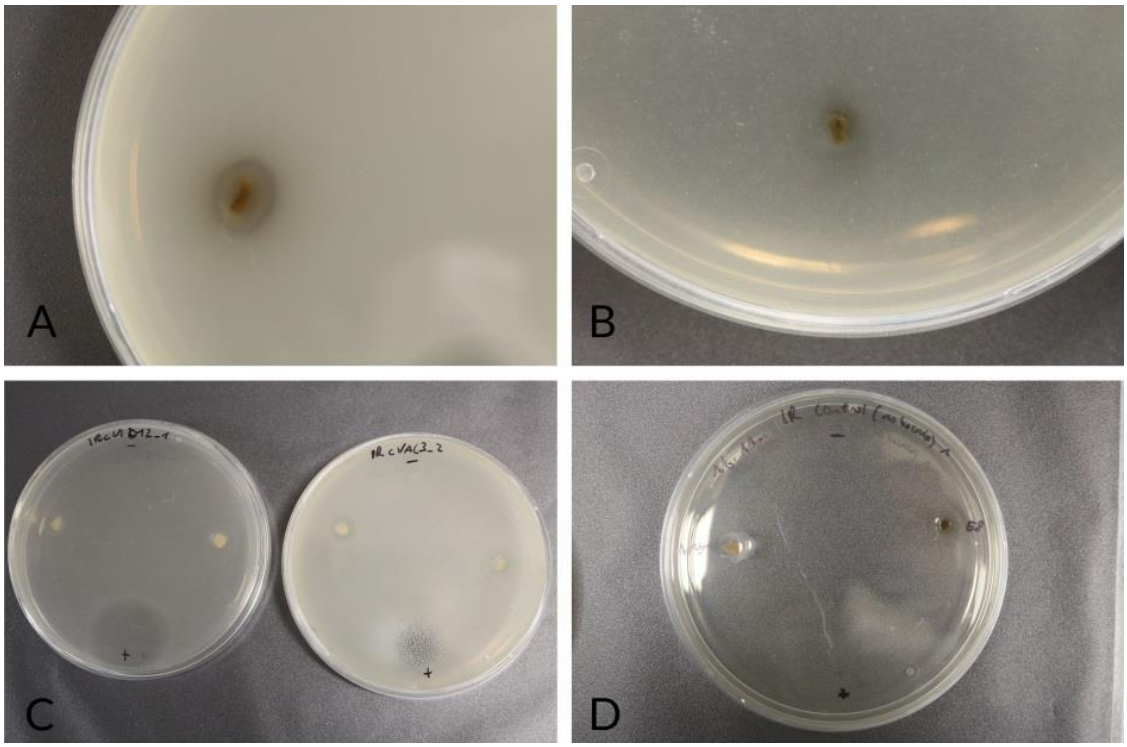

Fig. 12: A *Vibrio* from the Mediterranean plate with antimicrobial halo formation of *H. panicea*, **B** *Vibrio* from the Baltic plate with biostatic halo formation of *H. panicea*, **C** Comparison of density growth of bacteria (left *Vibrio* from the Baltic, right *Vibrio* from the Mediterranean), both plates with *Haliclona* sp. **D** Control plate without bacteria and with *H. panicea* (right) *and Haliclona sp*. (left).

The complete dataset contained halo size data for both species. It was used to compare the antimicrobial response of samples from each treatment (control, VM, VB) collected 6h vs 24h after treatment in aquaria. The complete dataset showed non-normal distribution (Shapiro-Wilks-test, control: p-value = 9.723e-06*; VM: p-value = 7.686e-05*; VB: p-value = 0.0009515*) but equal variances (Fligner-test (robust against non-normal distribution, non-parametric, p-value = 0.06097)). Each plate contained two technical replicates with one sponge piece on each side. There was no significant difference between them (t.test, p-value = 0.2886). Therefore both technical replicates were considered in the following tests. Differences between the three treatments are significant at 6h time point, between control and *Vibrio* from the Mediterranean (ANOVA 6h, F-value = 5.084, p-value= 0.0376 *, ANOVA 24h, F-Value= 4.887, p-value = 0.17, **Fig. 13**). Treatments are not significantly different from each other at the 24h time point. A trend is visible with an overall stronger reaction towards the *Vibrio* from the Mediterranean and at the 6h time point. The standard deviation indicates a strong variation in the data set.

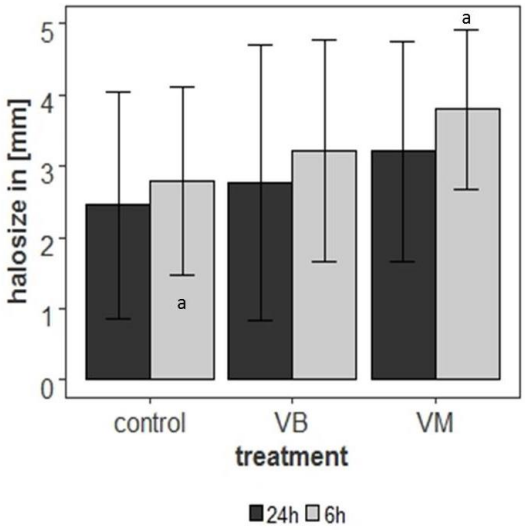

Fig. 13: **Results of Antimicrobial assay between treatments with complete data set (both species)** considering sampling time points (6h in grey, 24h in black). Halo formation (mm) was higher at 6h time point. Difference was significant between the Mediterranean *Vibrio* strain and the control (ASW) (a). (ANOVA 6h, p-value 0.0376 *) at 6h time point. p <.05, N=5.

I also analyzed the antimicrobial response of each sponge species separately. *H. panicea* dataset contained two biological replicates and *Haliclona* sp. dataset contained three biological replicates per treatment. Both datasets were non-normal distributed (Shapiro-Wilks-test, *Halichondria*: control: p-value = 0.001235*; VM: p-value = 0.1261; VB: p-value = 0.02875*; *Haliclona*: control: p-value = 0.002024*; VM: p-value = 0.0001709*; VB: p-value = 0.009656*). The *Halichondria* dataset had equal variances (Bartlett test: p-value = 0.7075). The *Haliclona* dataset had unequal variances (Bartlett test: p-value= 0.0001403*; Fligner test: p-value = 0.00115*). In both dataset there was no significant difference between the two sides of the plates. Both datasets showed strong variation on halosize, as indicated by the standard deviations. For statistical analysis results from both plate types were considered together to increase sample size.

Between the treatments of the *Halichondria* dataset a trend was visible towards a bigger halo size against the *Vibrio* from the Mediterranean, but there is strong variation in the control between 6h and 24h time point (**Fig. 14**). Two biological replicates were not enough samples for statistical analysis. The *Haliclona* dataset showed a significant difference at the 6h time point for the control to the *Vibrio* from the Mediterranean treatment and *Vibrio* from the Baltic treatment (Kruskal-Wallis 6h: p-value = 0.001073*; ANOVA 6h: p-value = 0.0448***; Fig. 14 B).** The variation within the treatments especially in the control of the *Halichondria* dataset was very strong. The *Haliclona* dataset was overall more consistent within the treatments (**Appendix 9.2.4**).

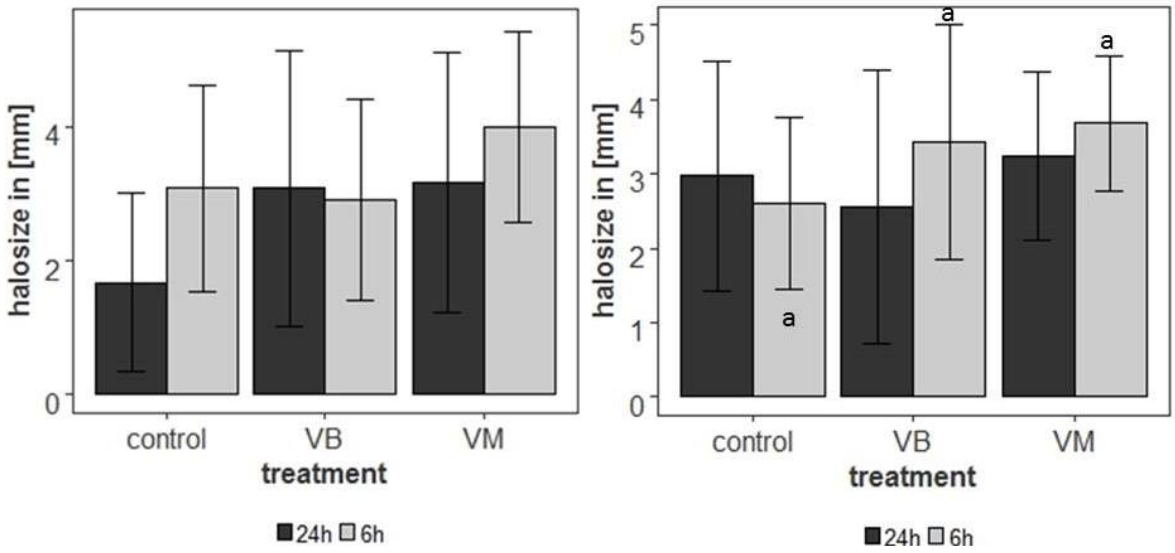

Fig.14: A Results of antimicrobial assay in samples from different treatments considering the two different sampling time points (6h in grey, 24h in black) in *Halichondria panicea* **(A)** and *Haliclona* sp **(B)**. a = significant difference of control (6h) to VB (6h) and VM (6h). p <.05, N=2(**A**), N=3(**B**).

5.5. RT-qPCR analysis

The target gene *hsp70* and the reference gene *actin* were successfully optimized for RT-qPCR analysis. Primer efficiencies of *actin* and *hsp70* were tested before running the experiment. The results were E=97.7% for *actin* (R^2=0.999) and E=97.1% for *hsp70* (R^2=0.998), what indicates a high primer efficiency. The primer efficiencies in the first immune response experiment (IR) with two biological replicates of *H. panicea* were 96.075% for *actin* (R^2= 0.994) and 91.268% for *hsp70* (R^2= 0.99). The primer efficiencies of the second immune response experiment (IR2) with three biological replicates of *H. panicea* were 85.585% for *actin* (R^2= 0.995) and 97,303% for *hsp70* (R^2= 0.997) (appendix **9.2.6**).

The samples of *H. panicea* from both immune experiments (IR and IR2) were combined to get five biological replicates per treatment. The data within each treatment was test for normality with Shapiro-Wilks-test (shapiro: VB: p-value = 0.03742*, VM: p-value = 0.3028). The QQ-Plot and the histogram indicate a non-normal distribution (**Fig. 15**). Under normality, the QQ-Plot is expected to be a linear curve with equal distribution of values and the histogram is expected to be a Gauß distribution. Homocedasticity was tested with Fligner-test (more robust against non-normal distribution). The Fligner-test was not significant (fligner: p-value = 0.07391), which indicates similar variances.

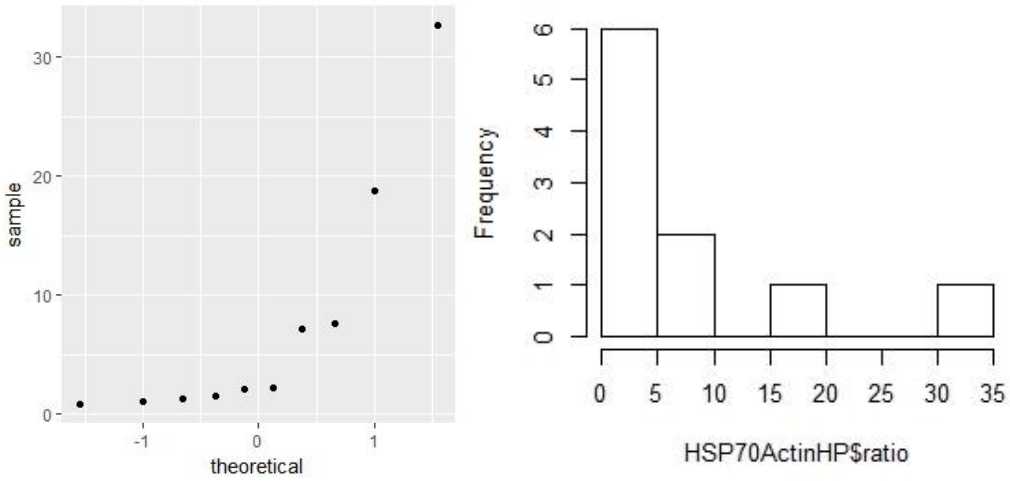

Fig. 15: The QQ-Plot (left) and the histogram (right) of the RT-qPCR dataset show a non-normal distribution.

A trend is visible towards a stronger expression of *hsp70* in the VM treatment, but the results were not significant (t.test: p-value = 0.1862, **Fig. 16 A**). Overall, the *hsp70* expression level was higher in the IR2 experiment and also the difference between the treatments was higher, but the results were not significant (t.test p-value = 0.08188, **Fig. 16 B**). Both plots show high standard deviations. In four of five individuals the treatment with VM showed an increase in the *hsp70* expression level compared to the reference gene *actin*, but the fold change values appear in a broad range (Pfaffl-method, ΔCt-values =1.29-32.72). A ratio of 1 indicates the same amount of expressed genes in the treatment as in the control. If considering the experiments separately, differences were only observed in the second experiment, whereas in the first one (IR) the ratios are very close to 1 (0.76-1.48).

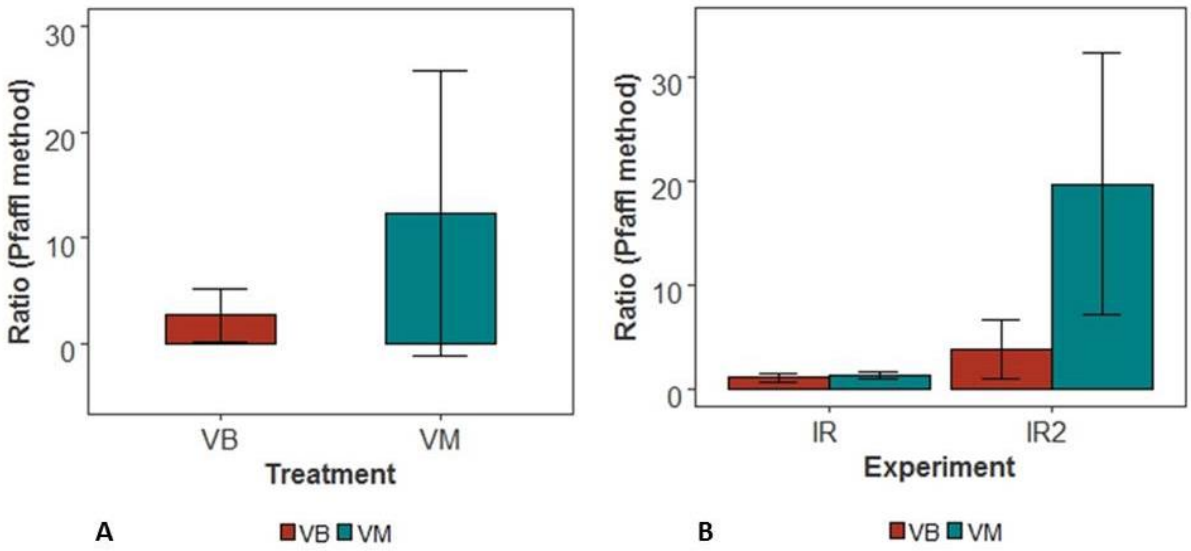

Fig. 16: Comparison of fold change ratio (by Pfaffl method) of the expression level of *hsp70* between treatments VB and VM in the whole dataset (**A**) and between treatments VB and VM separated by experiments (**B**). p <.05, N=5 (**A**), N=2 (IR), N=3(IR2) (**B**).

6. Discussion

6.1. Phylogenetic analysis

Based on macroscopic morphological features, I characterized the sponge individuals as "yellow morphotype" and "red morphotype". Phylogeny based on morphological features is often not precise enough, as many morphological features are similar between species. Genetic markers are a helpful tool to add to morphological classifications. However, no single ideal marker to classify all sponges exists and even the universal barcode markers COI mtDNA, 18s rRNA, 28s rRNA or the ITS-2 region showed different resolution level depending on the sponges investigated. For instance, 18S rRNA showed varying success for phylogenetic analysis in sponges, ranging from low phylogenetic signal (Szitenberg et al. 2013) to complete sponge classifications in Demospongiae (Redmond et al. 2013). Therefore, the combination of more than one molecular markers with morphological features is recommended (multilocus-based Sponge Identification Protocol (SIP) by Yang et al. (2017); Szitenberg et al. 2013).

The most reliable markers in my study were COI mtDNA and 18S rRNA, as they amplified sequences for both species in sufficient length and identified the two morphotypes as different species. 28S rRNA was reported as one of the most reliable molecular markers in sponge phylogeny (Szitenberg et al. 2013; Yang et al. 2017), but could not be successfully amplified for the candidate *Haliclona* sp. in my study. ITS-2 showed the lowest value of identification as both morphotypes were identified as the same species *H. panicea*, what confirms former studies claiming ITS-2 as insufficient marker especially for *Halicona* sp. (Yang et al. 2017; Redmond 2009), but contradicts reports of successful separation of sponge species based on ITS-2 (Erwin et al. 2011). By combining different molecular markers, I was able to identify the yellow morphotype as *Halichondria panicea* (18S rRNA, 28S rRNA, COI mtDNA) and the red morphotype as *Haliclona* sp. (18S rRNA, COI mtDNA). A clear identification was crucial, because the two species situation affected the experimental process and the primer design.

6.2. Aquaculture

The aquaculture of *H. panicea* was successfully performed according to the suspension method of Barthel & Theede (1986) in a flow-through system with only minor losses by molding. Barthel & Theede (1986) also tested a second method where sponge pieces were attached between glass slides, but the method was concluded to be less successful for survival rate. This second method was not actively performed in this study, but broken sponge pieces

of *H. panicea* attached independently to the bottom of the aquaria and were metabolically active. Thus, both growing conditions (suspension and attached to glass) were successful for survival of *H. panicea*. The suspension method set-up used for *H. panicea* was also successful for cultivating *Haliclona* sp. To my knowledge, this study is the first study describing a cultivation method for *Haliclona* sp. in an aquarium. A cultivation method with sponge transplants in a field aquaculture was performed with moderate success (Rosmiati et al. 2007).

H. panicea survived for 5 months in aquaculture, what is one month more than described in Barthel & Theede (1986) as long-term survival (4 months), but shorter than reported cultivation of *H. panicea* for one year (Müller 2003). The *Haliclona* sp. could be successfully kept for 12 weeks. However, compared to *H. panicea*, the survival rate was lower and therefore *Haliclona* sp. suits more to short-term maintenance. Based on the cultivation success, *H. panicea* seems to be a suitable candidate for a potential model organism, as the cultivation method is: easy to carry out and inexpensive (when flow-through system available, e.g. at GEOMAR), provides good long-term survival and cultured individuals suit for physiological and ecological experiments in the laboratory (Barthel & Theede 1986). The flow-through system provides more natural conditions and is highly recommended.

Before the experiment started, two specimen of *H. panicea* were observed to be covered with a grey biofilm, in its appearance similar to a fungal infection. Origins of the infection can be multiple, such as a pathogen encounter in the aquarium, pre-infected sponge individuals from the field or opportunistic microbes inside the sponge that turned pathogenic under aquaculture conditions. Similar infection events of *H. panicea* in the field are not described in literature, what could relate the issue to the sponge aquaculture. Infections in aquarium maintenance were observed before for *Ircinia* sp. and *Aplysina aerophoba* (Lucía Pita Galán, pers. comm.).The infection issue might be an interesting topic for further studies.

6.3. Immune response experiment

I hypothesized a differentiated immune reaction of the sponges towards the two different *Vibrio* strains and an increased immune response to the *Vibrio* strain from the Mediterranean (VM) (i.e. higher antimicrobial activity and differential gene expression), whereas the response to the *Vibrio* from the Baltic Sea (VB) stays similar to that in the control. The antimicrobial assay showed a stronger reaction to VM and a higher reaction at the 6h time point. The expression of the heat shock protein Hsp70 was analyzed with RT-qPCR in the

different treatments showing an upregulation of Hsp70 in VM treatment. Both analyses, the antimicrobial assay and the RT-qPCR, showed a differentiated reaction towards the *Vibrio* strains with a higher reaction to the VM, although the reaction was not consistent in all samples.

The antimicrobial activity of *H. panicea* and *Haliclona* sp. against both *Vibrio* strains indicates that the heat-killed *Vibrio* strains injected were taken as microbial challenge. Results suggested a trend towards a stronger reaction at the 6h time point in form of a wider halo formation. The time point corresponds with the study on immune priming in *Mnemiopsis leidyi* (Bolte et al. 2013). Other immunological studies in invertebrates confirm a high immune reaction within the first 24h hours (Pham et al. 2007; Sadd & Schmid-Hempel 2006; Zhang et al. 2011). Sampling time points can only reflect a snapshot of the physiological, molecular and behavioral changes after microbial encounter. Therefore, they affect the results of gene expression studies and should be carefully chosen. A stronger reaction at 6h time point may correspond with a higher gene expression level. Therefore, the 6h time point samples suited best for me to plan the proceedings of the experiment (RNA and DNA extraction and qPCR optimization). However, more than one sampling time point can help to provide a baseline for expressed genes and a time series on the reaction, e.g. monitoring of wound healing in cnidarians (Stewart et al. 2017). Thus, samples of the 24h sampling time point were stored for further analysis (**appendix 9.1.2**).

The antimicrobial assay and the RT-qPCR showed both strong variations in the dataset and may be related to small amount of replicates (2-3 biological replicates in *Halichondria* experiments, three biological replicates in *Haliclona* dataset). The antimicrobial activity in the control of the *Halichondria* dataset was also variable and suggests a general antimicrobial activity of the sponge by e.g. frequently expressed secondary metabolites or by compounds released by either microbes growing on the sponge surface or sponge-associated microbes inside the sponge (Schneemann et al. 2010; Kelman et al. 2001; Helber 2016). For future experiments it is recommended to run the experiments with more replicates per treatment or, if this is not possible because of logistics, analyze more samples of the same biological replicate.

Primer design for RT-qPCR analysis in the absence of a sequenced genome turned out to be a difficult challenge. It was not possible to optimize primers that worked for both sponge

species. Therefore, I focused on the sponge *H. panicea*, as it has been object of other studies within the research group. For this sponge, the cytoskeletal structure protein actin was successfully optimized as reference gene and the heat-shock protein Hsp70 gene as target. Ideally, more than one reference gene should be included to allow the evaluation of the gene as a reference of basal expression. In this study, the validation of actin has not been possible yet, but *actin* is a commonly used reference gene, stable under abiotic and biotic stress, as shown in other invertebrate studies (sponge: Webster et al. 2013; cnidaria: Rodriguez-Lanetty et al. 2008; Shimpi et al. 2016). Hsp70 is not involved in the signaling cascade of the immune reaction. However, expression changes upon microbial challenges were reported before (Brown et al. 2013; Brown & Rodriguez-Lanetty 2015; Zhou et al. 2010). In my study the RT-qPCR analysis, the upregulation of *hsp70* gene was higher with VM treatment in the IR2 experiment. The increased *hsp70* expression in VM treatment indicates a higher activity of the immune system compared to the VB treatment. The results encourage to persist the efforts to optimize genes involved in the immune signaling cascade.

Interestingly, the gene expression patterns (target gene expression in relation to reference) are not consistent in both immune experiments. Both immune experiments were performed under similar conditions. However, in the first experiment (IR) clones were used for the different treatments, whereas in the IR2 experiment individual specimens were applied. The application of clones in an experimental approach can be advantageous for reducing the genetic variation and increasing homogeneity of the dataset. Clonal sponge fragments remain metabolically active after cutting and recover quickly from wounding. However, in the case of low number of replicates, the use of clones may cause the genetic variation of samples within replicates of one treatment to be higher than the variation between the treatments and prevent the detection of differential gene expression. Furthermore, sponges used in the IR2 experiment spent three months longer in the aquaculture. Long-term aquaculture maintenance can cause changes in the bacterial community, as was reported for *H. panicea* (Müller 2003). This may increase vulnerability of sponges towards potential pathogens and therefore increase the reaction towards the applied bacteria, what could result in the increased *hsp70* expression level in the IR2 experiment.

The sponge species investigated here showed a specific reaction upon the challenge by different *Vibrio* stains. In the antimicrobial assay, both sponges showed higher intensity and antibiotic response against the VM treatment than to VB (biostatic response). This implies a

specific reaction of the sponges towards the different *Vibrio* stains. To date, specificity on bacterial strains was mainly reported for higher invertebrates, such as insects (Roth et al. 2009) or crustaceans (reviewed in Schulenburg et al. 2007). However, the immune priming experiment of Bolte et al. 2013 with *Mnemiopsis leidyi* suggests specificity also in basal metazoans. The lower reaction against VB could indicate that the sponges just recognized the strain as non-pathogenic or, considering the hypothesis of immune memory in invertebrates, encountered this *Vibrio* strain before the experiment, as *H. panicea* was found in the same habitat (HELCOM Red List Biotope Expert Group 2013).

Furthermore, the specificity can be designated by the *Vibrio* strains themselves by variable virulence. The family of *Vibrionaceae* is highly diverse and members of the genus *Vibrio* spp. are not necessarily pathogenic (Thompson et al. 2004). Some of them live even in symbiotic relationships, e.g. the bioluminescent *Vibrio fischeri* with the Hawaiian bobtail squid *Euprymna scolopes* (Nyholm & McFall-Ngai 2004). In contrast, other members are known for their high pathogeny to, e.g. humans, such as *Vibrio cholera* (Thompson et al. 2004). Their pathogenic potential is tightly coupled to environmental conditions (temperature, availability of iron), cell density (expression of virulence genes via quorum sensing), motility and chemotaxis (Thompson et al. 2004). Some *Vibrio* strains can gain or increase their pathogeny by taking up virulent plasmids (Roux et al. 2011). The *Vibrio* strains used in this study were not further classified. Therefore their virulence might vary from each other and cause different reactions of the sponges.

To distinguish if the stronger reaction of the sponges towards the *Vibrio* from the Mediterranean is caused by immune memory of the Baltic Sea sponges to VB or by the high virulence of the VM, a similar experimental design applied to a Mediterranean sponge could provide answers. If the reaction of the Mediterranean sponge is higher to VM than to VB, the different immune reaction is more likely provoked by the virulence of VM. If the reaction of the Mediterranean sponge is more intense towards the exogenous Vibrio (in their case VB) this could indicate adaption or immune memory of the sponges. Important for the concept of immune memory is a specific response to repeated infections compared to first encounter. Specificity and immune memory can be further investigated by homologous vs. heterologous treatment (Bolte et al. 2013) In this type of experiments, individuals are exposed to twice the same *Vibrio* (homologous treatment) or to two different *Vibrio* strains (heterologous treatment). Under the hypothesis of immune memory, I expect that the sponge will react

different towards the two *Vibrio* strains and the two treatments detectable in differentially expressed genes. Depending on the investigated genes, I expect a downregulation consistent with the evolutionary concept of reducing costs and self-damage of immune defense or an upregulation of specific genes due to immune memory (quicker and more effective response upon second injection).

The long-term cultivation in an aquaculture system was successfully established for *H. panicea*, what will help to provide sponges for experiments under controlled conditions. Furthermore, this study provides important knowledge on specificity in *H. panicea* towards different *Vibrio* strains. Specificity in sponges unravels potential mechanisms for controlling microbial communities in these basal metazoans and can build a baseline for more complex processes, such as immune memory in sponges or symbiosis. The presence of specific reactions towards bacteria in these basal metazoans suggests highly conserved pathways for animal-microbe interactions. Further studies should focus on developing more genetic markers involved in the host mechanism to respond to microbes.

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9. Appendix

9.1. Additional samples

9.1.1. Bacterioplankton samples for 16S rRNA analysis

To identify which groups dominate the bacterioplankton 1L of the inflowing aquarium water and the Kieler Förde water were filtered on a 0.22 µm filter and stored at -80°C. Diversity will be measured by 16S rRNA amplification of DNA. The protocol for sequencing has not been defined yet.

9.1.2. Sponge material in RNAlater

Samples were taken for 24h time point close to the injection zone and samples for both time points were taken distant from the injection zone (compare local or general immune reaction). The samples are not analyzed yet, due to optimization procedure of the 6h time point samples.

9.2. Supplementary Material

9.2.1. Fluorescein results

Table 5: Fluorescein results (taken 1 day before each experiment start)

Sponge/Experiment	FL test	Sponge/Experiment	FL test	Sponge/Experiment	FL test
A1/IR	+	D12/IR	+	G1/IR2	+
B2/IR	+	E13/IR	+	H2/IR2	+
C3/IR	+	A15/IR	+	I3/IR2	+
D4/IR	+	C16/IR	+	J4/IR2	+
A5/IR	+	B17/IR	+	K5/IR2	+
B6/IR	+	E18/IR	+	L6/IR2	+
D7/IR	+			M7/IR2	+
E8/IR	+			N8/IR2	+
C11/IR	+			O9/IR2	+

9.2.2. Light intensity and temperature in IR2 experiment (HOBO logger)

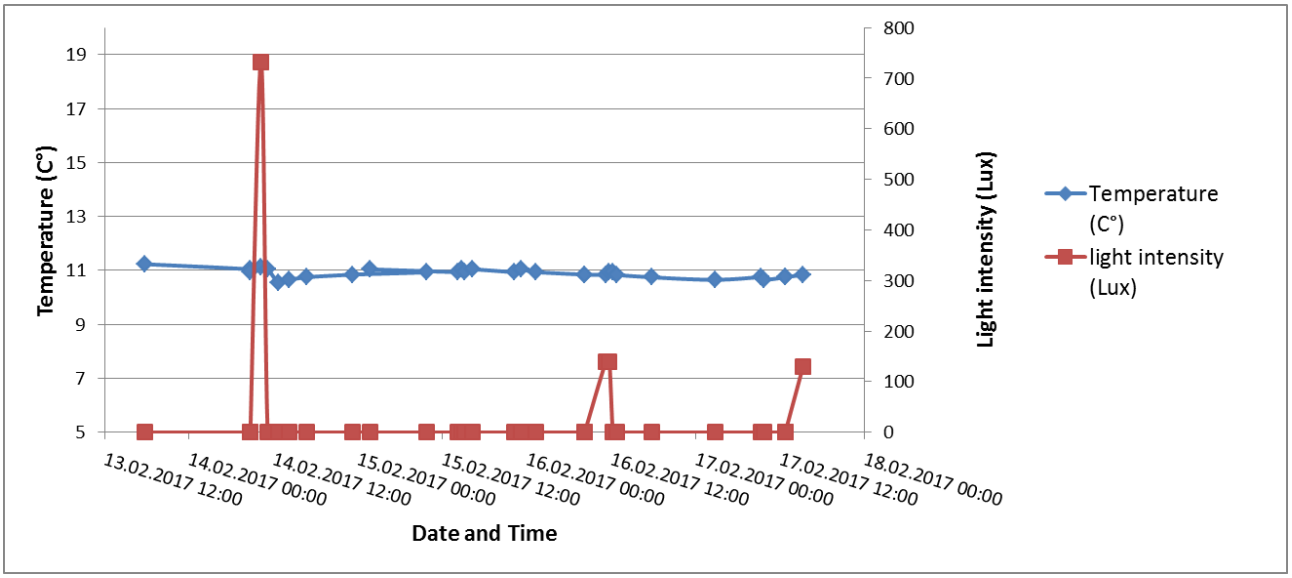


Fig. 17: Temperature and light intensity of IR2 experiment. Peaks in Light intensity correspond with working hours at aquarium where light was switched on.

9.2.3. Oxygen, temperature, pH, and salinity in IR and IR2 experiment

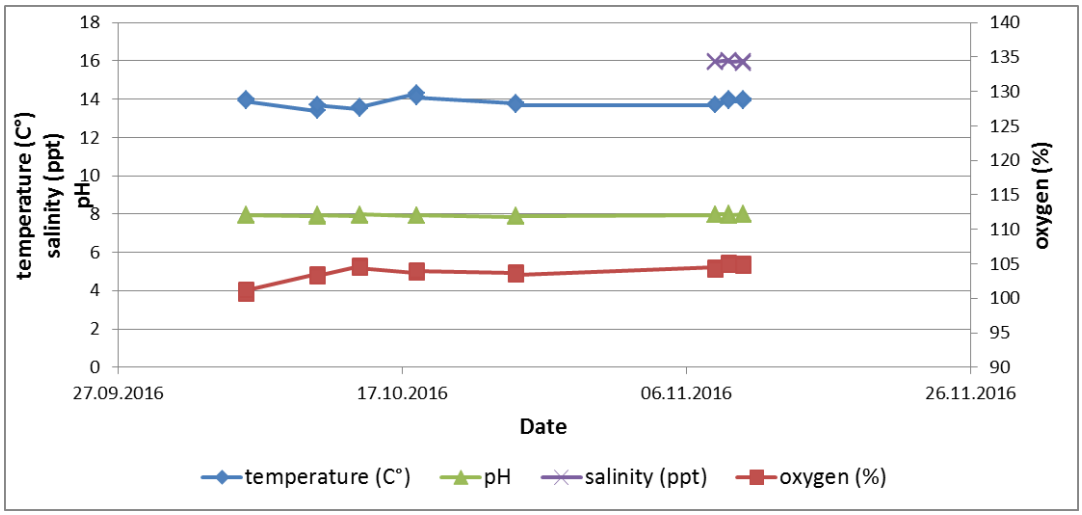


Fig. 18: Oxygen, temperature, pH and salinity results in IR experiment

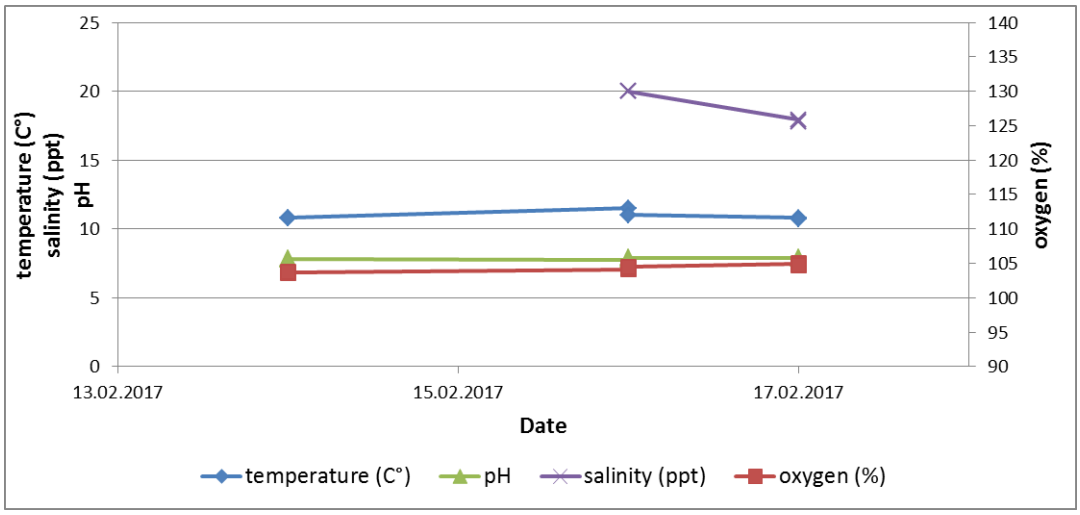


Fig. 19: Oxygen, temperature, pH and salinity results in IR2 experiment

9.2.4. Results of antimicrobial assay within treatments

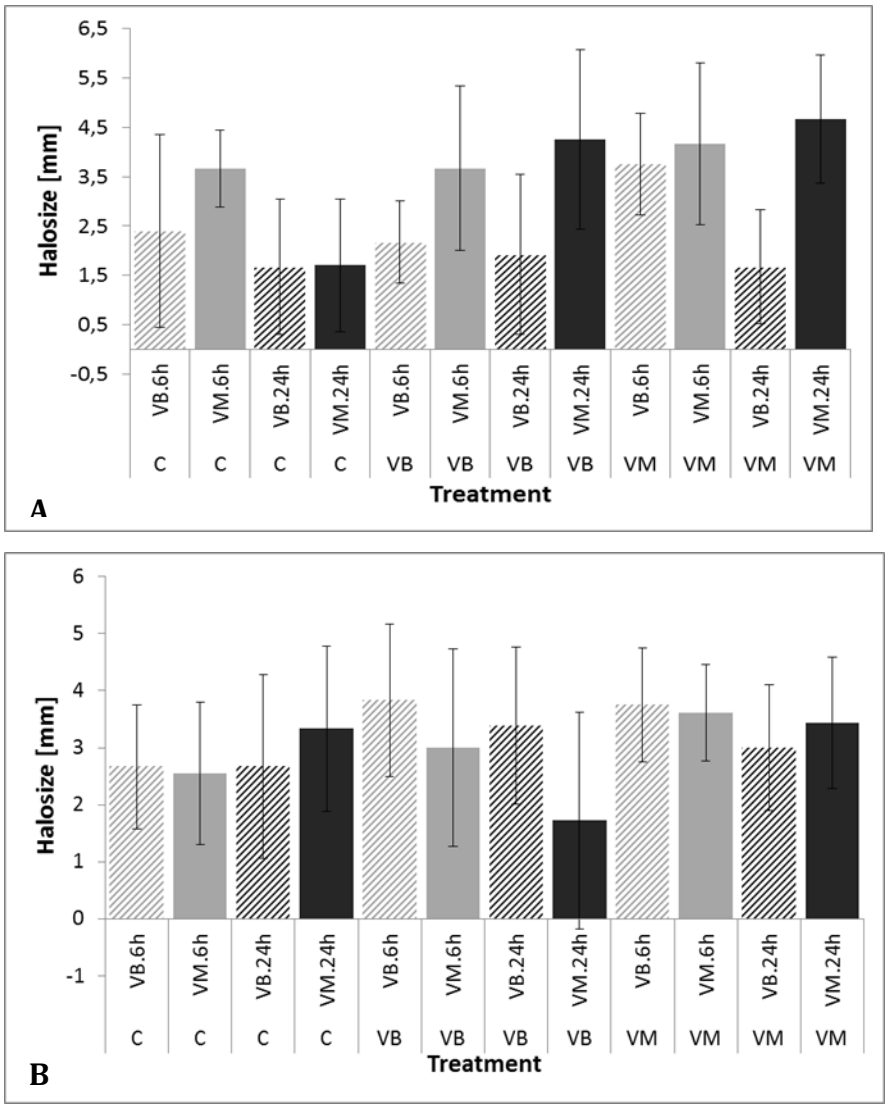


Fig. 20: Results of antimicrobial assay within treatments of *H. panicea* (**A**) and *Haliclona sp*. (**B**) considering treatment, time point and bacteria on plates. Strong variation in controls of *H. panicea*. *Haliclona* sp. overall more consistent.

9.2.5. Genbank ID of species in phylogenetic trees (18s rRNA, ITS-2 and COI mtDNA)

Molecular marker	**Species**	**Accession No. NCBI Gen-bank**
18s rRNA	*Actinia equina*	AJ133552.1
18s rRNA	*Aiptasia pulchella*	AY297437.1
18s rRNA	*Amphimedon compressa*	EU702409.1
18s rRNA	*Aplysina aerophoba*	AY591799.1
18s rRNA	*Axechina raspailioides*	EF092263
18s rRNA	*Cliona sp.*	KC902056.1
18s rRNA	*Didiscus sp.*	EF094549
18s rRNA	*Halichondria panicea*	KF699110.1
18s rRNA	*Haliclona cinerea*	DQ927306.1
18s rRNA	*Haliclona mediterranea*	AY348879.1
18s rRNA	*Haliclona oculata*	AY734450.1
18s rRNA	*Haliclona sp.*	AY734444.1
18s rRNA	*Mus musculus*	NR_003278.3
18s rRNA	*Mnemiopsis leidyi*	AF293700.1
18s rRNA	*Myrmekioderma sp.*	GQ466053
18s rRNA	*Nematostella vectensis*	AF254382.1
18s rRNA	*Oceanapia sp.*	DQ927317.1
18s rRNA	*Petrosia sp.*	DQ927320.1
18s rRNA	*Pleurobrachia pileus*	AF293678.1
18s rRNA	*Reniochalina stalagmitis*	EF092272
18s rRNA	*Tethya sp.*	KC901956.1
18s rRNA	*Xestospongia muta*	AY621510.1
ITS-2	*Actinia bermudensis*	JN118562.1
ITS-2	*Actinia equina*	DQ831298.1
ITS-2	*Aiolochroia crassa*	AY591798.1
ITS-2	*Aiptasia mutabilis*	DQ831297.1
ITS-2	*Aplysina aerophoba*	AY591786.1
ITS-2	*Cliona viridis*	AF062606.1
ITS-2	*Halichondria panicea*	AF062607.1

ITS-2	*Haliclona tubifera*	JF824785.1
ITS-2	*Mnemiopsis leidyi*	AF293700.1
ITS-2	*Pleurobrachia bachei*	AF293677.1
ITS-2	*Stichopus monotuberculatus*	HM162897.1
COI mtDNA	*Acropora sp.*	JQ920466.1
COI mtDNA	*Amphimedon compressa*	EF519560.1
COI mtDNA	*Aplysina cauliformis*	EF519569.1
COI mtDNA	*Cliona sp.*	AM076983.1
COI mtDNA	*Halichondria magniconulosa*	EF519616.1
COI mtDNA	*Halichondria melanodocia*	EF519617.1
COI mtDNA	*Halichondria panicea*	KC869423.1
COI mtDNA	*Halichondria sp.*	EF217339.1
COI mtDNA	*Haliclona sp.*	LC126249.1
COI mtDNA	*Haliclona toxius*	LC126248.1
COI mtDNA	*Haliclona tubifera*	EF519624.1
COI mtDNA	*Ircinia campana*	EF519637.1
COI mtDNA	*Nematostella vectensis*	DQ538492.1
COI mtDNA	*Oceanapia sp.*	AY561967.1
COI mtDNA	*Petrosia sp.*	JN242220.1
COI mtDNA	*Stichopus monotuberculatus*	KC424518.1

9.2.6. Standard curves for primers used in RT-qPCR

9.2.6.1. Actin (reference gene)

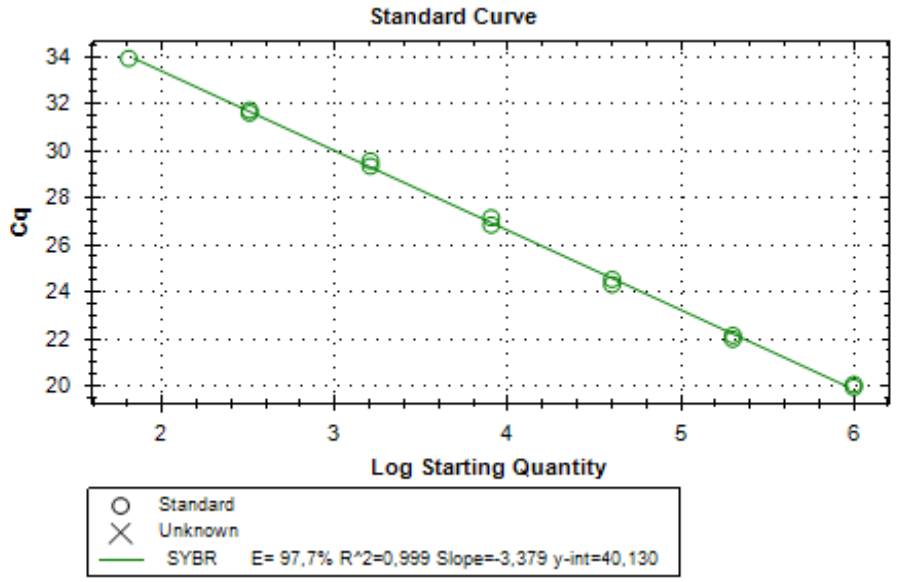


Fig. 21: Standard curve Actin

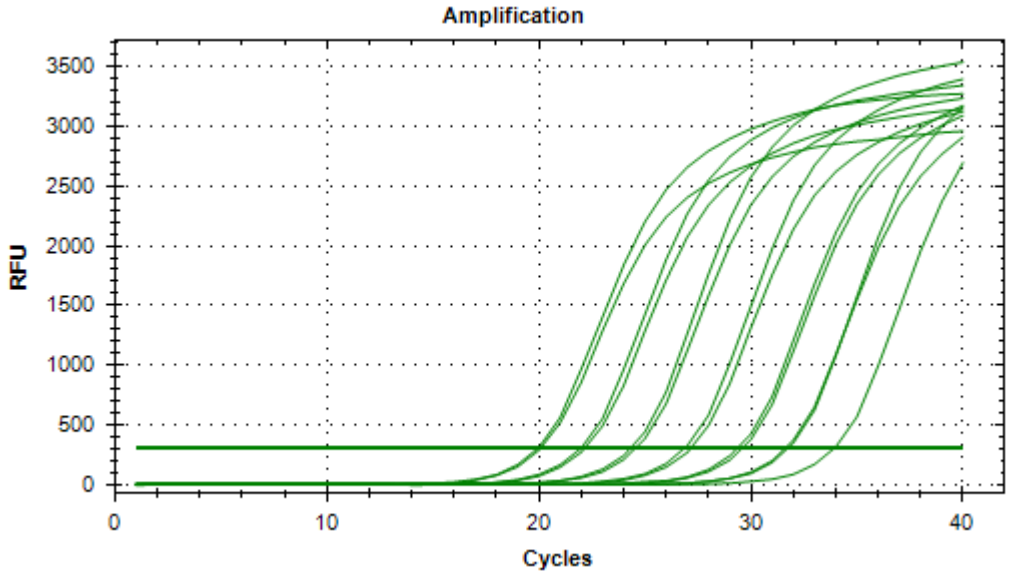


Fig. 22: Amplification Plot of standard curve Actin

9.2.6.2. Hsp70 (target gene)

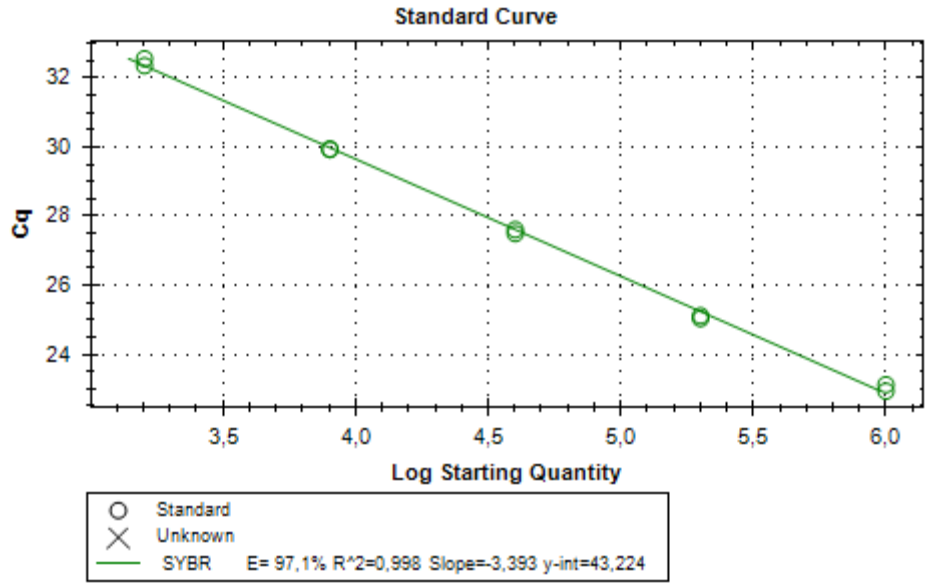


Fig. 23: Standard curve Hsp70

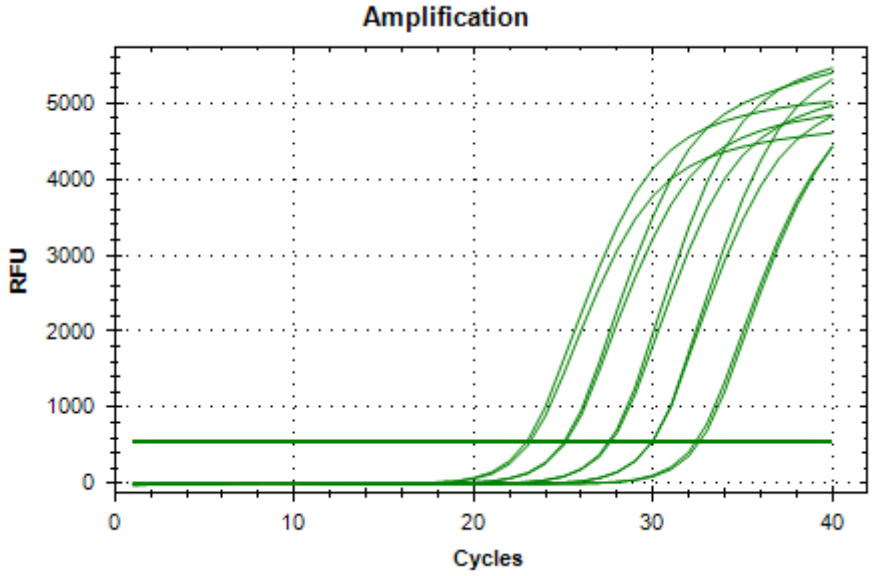


Fig. 24: Amplification plot standard curve Hsp70.

9.3. Procedure Protocols

9.3.1. NUCLEIC ACID EXTRACTION FOR TRANSCRIPTOMICS **with AllPrep DNA/RNA mini kit (Qiagen)** August 1, 2015

Protocol based on Moitinho-Silva et al. (2014) Environmental Microbiology, adjusted by Lucía Pita Galán

AllPrep DNA/RNA mini kit

For sponge samples stored in RNAlater.

Only max. 12 extractions at a time (maximum 3 different biological samples) to reduce time of extraction.

Materials and reagents

RNaseZap Ambion	RNase decontamination of working surfaces
Gloves	
Blades	Excisions of collected tissues
Forceps	Excisions of collected tissues
Petri dish	To put there the tissue cuts
Eppendorf tubes (1.5mL)	
Precision scale	Calculate the amount of tissue (wet weight) used for the extraction
FastPrep homogeneizer	Tissue lysis
Lysing matrix-E tubes (MP Biomedicals)	2mL tubes for cell lysis. This tube contains ceramic (1.4mm) and silica spheres (0.1mm) as well as glass beads (4mm) to mechanical cell disruption
AllPrep DNA/RNA mini kit (qiagen)	DNA/RNAextraction. Check AW1 and AW2 have no precipitate. If so, dissolved at 50°C.
1% ß-mercaptoethanol (14.3M) in RLT**plus** buffer (qiagen kit)	Tissue lysis and RNA stabilization during lysis. Prepare in the hood the quantity need for the week

96-100% ethanol	Add to Qiagen reagents if necessary
70% ethanol	Step 6 of the Qiagen protocol
SUPERase-In (20 U/uL, Ambion USA) or RNAsin (Promega)	RNase inhibition
RQ1 RNase-Free DNase (20 U/uL; Promega, Germany) and stop buffer. Or DNase from SIGMA	Remove any rest of DNA from RNA extract
Blue,yellow and white-**filter** tips	
ice	
Boxes for samples storage	

Other notes:
Beta-mercaptoethanol is very toxic, work always in the hood.

0. BEFORE BEGINNING

1. Check all the reagents. If necessary, prepare 1%beta-mercaptoethanol in RLTplus buffer (see below).
2. Print Excel sheet for annotation of weights (or prepare table in lab book).
3. Clean hood, bench, any surface and instrument to be used with RNAse Zap
4. Take the sample from the freezer and thaw on ice.

Prepare a solution of 1% ß-mercaptoethanol (14.3M) in RLT**plus** buffer (Qiagen kit) in the hood (prepare the solution I need for the week). Taking into account that: 1 sample = 6 uL ß-mercaptoethanol (14.3M) + 594 uL RLT**plus** buffer.

A. PREPARING TISSUE FOR EXTRACTION

1. Label n 2mL Lysing matrix E tubes (MP Biomedicals) for n extractions and put them on ice.
2. Bring to the precision scales forceps, scalpel, petri plate, gloves, RNase Zap, samples and tubes.

3. Select one tissue sample. Cut a piece of 1cm3 from the sample (in a Petri dish by using forceps and blades (everything should be previously treated with RNase Zap). **Cut the fragment in smaller pieces**. **Tissue excisions should include pinacoderm, mesohyl and choanoderm.**

4. Tare the lysis tube. Load the tube with tissue pieces until having **30-50 mg*** of tissue. Annotate tissue weight (wet weight) in RNAextraction yield sheet.

***Optimization process**: For *Halichondria panicea* samples 50-60mg worked best.

B. CELL LYSIS

According to Giles et al. (2013)

1. Add 600 µL of 1% ß-mercaptoethanol RLTplus buffer to each tube **under the hood**.
2. Cells disrupted in the homogeneizer PowerLyzer ^{TM} 24 (MoBio) 30 sec at speed of 3000
3. Centrifuge for 10 min, máx speed.

C. DNA/RNA EXTRACTION ACCORDING TO MANUFACTER´S PROTOCOL (modifications **in bold**)

4. Carefully remove the supernatant by pipetting, and transfer it ot the AllPrep DNA spin column placed in a 2mL collection tube (supplied). Close the lid gently, and centrifuge for 30s at ≥8000g (≥ 10000rpm).

5. Place the AllPrepDNA spin column in a new collection tube (supplied), and store at RT or at 4°C for later DNA purification (steps 14-17). USE THE FLOW-THROUGH FOR RNA PURIFICATION STEPS (6-13).

Total RNA purification

6. Add 1 volume (usually 370 µL) of 70% ethanol to the flowthrough from step 5, and mix well by pipetting. Do not centrifuge! Proceed immediately to step 7.

7. Transfer up to 700 µL of the sample, including any precipitate, to an RNeasy spin column placed in a 2mL collection tube (supplied). Close the lid gently, and centrifuge for **30**s at ≥8000 g (≥10000 rpm). Discard the flowthrough (reuse the collection tube in step 8).

If the sample volume exceeds 700uL, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

8. Add 700 µL of Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for **30**s at ≥8000 g (≥10000 rpm) to wash the spin column membrane. Discard the flow-through (reuse the collection tube in step 9).

9. Add 500 µL Buffer RPE to RNeasy spin column. Close the lid gently, and centrifuge for **30**s at ≥8000 g (≥10000 rpm) to wash the spin column membrane. Discard the flow-through (reuse the collection tube in step 10).

10. Add 500 µL Buffer RPE to RNeasy spin column. Close the lid gently, and centrifuge for 2 min* at ≥ 8000 g (≥ 10000 rpm) to wash the spin column membrane. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through.

11. Place the RNeasy spin column in a new 2mL collection tube (supplied), and discard the old collection tube with the flowthrough. Centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 10.

12. Place the RNeasy spin column in a new 1.5mL collection tube (supplied). Add 50 µL RNase-free water directly to the spin column membrane. Close the lid gently **and let it on the bench for 10 min.** Centrifuge for 1 min at ≥8000 g (≥10000 rpm) to elute the RNA.

13. **Repeat step 12 using again 50 µL of RNase-free water and let 1 min on bench.**

Keep extracts always on ice!

Take a 6 µL aliquot (for Nanodrop and Experion analysis).

RNase inhibition

Add 10 µL (=0.1 Volume of RNA) Anti-RNase (Ambion) to 100µL RNA

Mix gently

Nuclease Treatment (Kit: DNA-free™ Kit DNase Treatment and Removal Reagents)

Add 0.1 volume 10X DNase I Buffer and 1 µL rDNase I to the RNA, and

mix gently.

Incubate at 37°C for 20 min

Add resuspended DNase Inactivation Reagent (typically 0.1 volume)

and mix well.

Incubate 2min at room temperature, mixing occasionally.

Centrifuge at 10,000 × g for 1.5min and transfer the RNA to a fresh tube.

Genomic DNA purification:

13. Add 500 uL of Buffer AW1 to the AllPrep DNA spin column from step 5. Close the lid gently, and centrifuge for **30**s at ≥8000 g (≥10000 rpm). Discard the flow-through (reuse the spin column in step 15).

14. Add 500 uL Buffer AW2 to AllPrep DNA spin column. Close the lid gently, and centrifuge for 2 min at full speed to wash the spin column membrane. After centrifugation, carefully remove the AllPrep DNA spin column from the collection tube.

15. Repeat step 14.

16. Place the AllPrep DNA spin column in a new 1.5 mL collection tube (supplied). Add **50uL** Buffer EB directly to the spin column membrane and close the lid. Incubate at room temperature for **5** min, and then centrifuge for 1 min at ≥8000 g (≥10000 rpm) to elute the DNA.

17. Repeat step 16. Let 1 min on bench

D. NUCLEIC ACID QUANTIFICATION

D.1. Quantification in NanoDrop:

- For each extraction, annotate: concentration (ng/uL), A260, A260/280 ratio, A260/230.

Nanodrop: Clean the sensor. Choose "Nucleic acids". Set the type of nucleic acid (RNA or DNA).

- Use water as blank for RNA quantification. Use EB buffer as blank for DNA quantification.

9.3.2. cDNA transcription (iScript™ Select cDNA Synthesis Kit)

1. Thaw all components except iScript reverse transcriptase. Mix thoroughly and briefly centrifuge to collect contents to the bottom of the tube before using. Place components on ice.

2. Add the following components to a 0.2 ml PCR tube or each well of a 96-well PCR reaction plate on ice:

Components	**Volume**
Nuclease-free water	Variable
5x iScript select reaction mix	4 µl
Oligo(dT)20 primer or random primer	2 µl
RNA sample (1 pg to 1 µg total RNA)	Variable
iScript reverse transcriptase	1 µl
Total	20 µl

Note: for multiple reactions, prepare a master mix with the above components, except RNA, and then dispense to each reaction.

3. Mix gently and incubate as follows:
For oligo(dT)-primed cDNA reactions, incubate for 60–90 min at 42°C.
For random-primed cDNA reactions, incubate for 5 min at 25°C, then 30 min at 42°C.

4. Incubate at 85°C for 5 min to heat-inactivate the reverse transcriptase.

5. Store cDNA product at –20°C to +4°C.

6. The resulting cDNA product can be used directly for PCR amplification. Typically, one-tenth (2 µl) of the first-strand reaction provides sufficient target for most PCR applications. Optionally, the cDNA can be diluted in TE buffer [10 mM Tris (pH 8.0), 0.1 mM EDTA] for addition of larger volumes (5–10 µl) to PCR reactions.

9.3.3. FIXATION FOR FLOW CYTOMETRY OF PHYTOPLANKTON AND BACTERIA IN SEAWATER

-Based on Pep Gasol (ICM-CSIC Barcelona, Spain) and Laura Rix (GEOMAR) protocols.

Notes:

(i) Prepare fresh and store in the fridge to use within a week (even up to several weeks). Or prepare bigger amounts, aliquot in tubes, freeze them at -80°C and store at -20°C (to use up to several months).

(ii) The chemical compounds used for the fixative are toxic. Work always under the fume hood.

(iii) For heating and stirring under the hood, use one of the devices from upstairs, but ask in advance Tanja, Ignacio and Álvaro.

Preparation of fixative-protocol for 20 mL:

1) Weight 2 g of paraformaldehyde (SIGMA P6148) under the fume hood.
2) Place in 17.6 mL of miliQ water in a beaker covered with Parafilm (to reduce water loss through evaporation) and with a magnetic fly. Stir vigorously at 70°C for at least 2h under the fume hood, until the paraformaldehyde dissolves and saturates the water.
3) Add small amounts of 1N NaOH drop-wise until the solution becomes clear.
4) Let the solution cool to RT
5) Adjust the pH to 7.4 with 1N HCl. Add the HCl drop-wise.
6) Add 2 mL of 1 x PBS
7) Add 0.4 mL of Glutaraldehyde 25%
8) Filter through 0.2 µm polycarbonate syringe into a Falcon tube
9) Store in the fridge*

*for big amounts and long storage: Aliquot the fixative into tubes, freeze them at the -80°C and store them at -20°C.

Sampling and sample fixation:

1) Label 15 mL sterile falcon tubes and 2 mL cryovials (final sample) in advance. Prepare duplicate cryovials from each seawater sample.
2) Collect seawater sample (e.g. from bucket) or directly into the falcon tube. Rinse the recipient three times with the water to be sampled. Then collect the water and proceed as soon as feasible. (ideally, keep the tubes cold).

3) Under the hood, pipette 1.8 mL of seawater sample into 2 mL cryovials.
4) Then pipette 0.2 mL of fixative (10%) into the vial. This yields a final 1% fixative concentration. Remember to filter before use.
5) Mix the vials and let them stand at room temperature for not least than 10 min (and max. 30 min).
6) Quick freeze the vial in liquid nitrogen and store at -80°C. If samples are not for virus, then tubes can be directly freeze at -80°C.

9.3.4. Experion™(Experion™ RNA StdSens Starter Kit)

Protocol based on manufacture´s instruction, adapted to more straightforward procedure in our lab.

For total RNA derived from nucleic acid extraction that showed good quality in Nanodrop. (aliquots before RNase inhibitor and DNase treatments).

Materials and reagents

	Utility
RNaseZap Ambion	RNase decontamination of working surfaces
Gloves	
RNase-free tubes (0.5 mL)	For samples and ladder
RNA StdSens Experion kit (Bio-Rad)	Include chips (RT, in Christina's drawer); chemical reagents and filter columns (in the fridge). And RNA ladder (at -80°C)
Total RNA extracts	Use the aliquots!
DEPC-treated water	For cleaning (take a 50 mL Falcon tube)
RNase X plus	For cleaning (take a 50mL Falcon tube). Check it is transparent! Sometimes it oxidizes and looks yellow…
Thermocycler	Denaturation step
Blue,yellow and white-**filter** tips	
ice	
Experion stations	3 stations: priming, vortex and electrophoresis. In Botanik I
A pen-drive	To save the results

Procedure overview

1. Equilibrate reagents to room temperature for **20 min** (except RNA ladder and RNA aliquots, that should be thaw on ice).
2. Filter gel (**10 min**). Add stain to filtered gel. Keep the rest of filter gel (9uL of non-stained filter gel are needed to load G well in the chip). Filtered gel is stable for 1 month if kept protected from light at 4°C.
3. Prepare an aliquot of buffer, "B", (15 x 5uL = 75 uL for one chip is enough).
4. Take **2µL** of RNA aliquots into new 0.5 mL tubes labeled 1-12 as they are going to be load in the chip

5. Heat RNA ladder and samples in thermocycler for **2 min** at 70°C (heat lid ON)-denaturation. Then keep on ice (minimum **5 min**).
6. Clean electrodes before run (x 3)
7. During second cleaning, prime chip with gel-stain solution.
8. During third cleaning, load and vortex chip.
9. Run **RNA StdSens** analysis protocol on the Experion electrophoresis. The one for **eukaryotic** samples
10. Clean instrument electrodes.

Essential practices

Aliquot RNA ladder (2uL) in 0.5 mL RNase-free tubes and store at -80°C. Store all the other chemical reagents of the kit at 4°C when not in use. Clean surfaces with RNaseZap. Before using, allow kit reagents to equilibrate to room temperature with the exception of the RNA ladder (15-20min). Vortex and briefly centrifuge all kit reagents before use. Protect RNA stain and gel-stain solution from light.

Always heat-denature the RNA ladder and RNA samples immediately before use and keep on ice until loading the chip.

Run the loaded chip within 5 min of loading to prevent excessive evaporation

A. EQUILIBRATING KIT REAGENTS

1. Remove the RNA Stain (**blue cap**), RNA loading buffer (**yellow cap**), and 1 tube of RNA gel (**green cap**) from storage and equilibrate to room temperature for ~ 15–20 minutes. **Keep the stain covered at all times to avoid exposure to light**.
   Note: If filtered gel was previously prepared, remove it from storage and equilibrate as detailed above.
2. Vortex the contents of each tube and briefly centrifuge to bring the solutions to the bottom of the tubes. Make sure the DMSO in the stain is completely thawed before proceeding.

Filtered gel is stable for 1 month, if kept at 4°C and avoid light exposure

Remove RNA ladder and total RNA extract aliquots from -80°C and thaw on ice.

B. PREPARING THE GEL STAIN

Note: Skip steps 1 and 2 if filtered gel is already available.

1. Pipet 600 µl RNA gel (**green cap**) into a spin filter tube.
2. Centrifuge the gel at 1,500 x g for 10 minutes. Confirm that all of the gel has passed through the filter and then discard the filter.
    - **Note**: Use the filtered gel within 4 weeks of preparation. After 4 weeks, the filtered gel should be re-filtered and can be reused.
3. Pipet 65 µl filtered gel into an RNase-free microcentrifuge tube. Add 1 µl RNA stain to the tube. Briefly vortex the solution. Keep the gel-stain (GS) solution protected from light.
    - This is enough gel-stain solution for 3 chips. Increase the amount of gel stain, if required, by using a 65:1 ratio of gel and stain. Fress gel-stain solution should be prepared daily.
4. Cap the RNA stain tightly, since DMSO is highly hygroscopic, and store it in the dark.

C. PREPARING THE SAMPLES AND RNA LADDER

RNA ladder aliquots are ready to use: Each aliquot of RNA ladder contains already the 2uL needed for one chip.

Prepare all samples by pipetting 3 uL sample into RNase-free 0.5 mL tube. If RNA concentration is high (>400 ng/uL) consider dilute the extract in RNase-free water. Denature the ladder and samples for 2 minutes at 70°C in the thermocycler (big wells). Cool the denatured ladder and samples by immediately placing the tubes on ice for 5 minutes. Spin down the ladder and samples in a microcentrifuge for 3-5 sec.

D. CLEANING THE REAGENTS BEFORE THE RUN

1. Fill a cleaning chip with 800 µl Experion electrode cleaner. Check to make sure there are no air bubbles trapped in the reservoir. Gently tap the side of the cleaning chip to dispel any bubbles. Label this cleaning chip as the electrode cleaner chip.
2. Open the lid of the electrophoresis station and place the chip on the platform.
3. Close the lid and leave the chip in the instrument for 2 minutes.
4. Fill a separate cleaning chip with 800 µl DEPC-treated water. Label this cleaning chip as the DEPC water chip.

Note: If this cleaning chip is being used for the first time, treat the chip with the Experion electrode cleaner to remove any RNase contamination prior to use. To do this, completely fill a new cleaning chip with Experion electrode cleaner, let sit for 5 minutes, discard the solution, and then thoroughly rinse (4–5 times) the chip with DEPC-treated water.

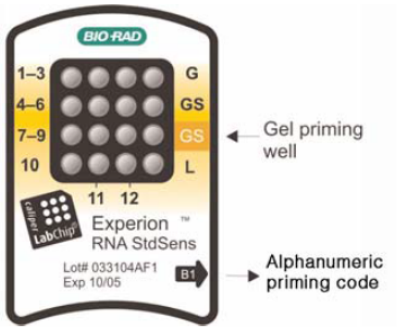
5. Open the lid and remove the cleaning chip containing the electrode cleaner; replace it with the chip containing the DEPC-treated water.
6. Close the lid and leave the chip in the instrument for 5 minutes to rinse the electrodes.

During water cleaning step 6, prime the chip (section E) and load the chip (section F)

7. Replace the DEPC-treated water in the DEPC water chip and repeat the rinse step for 60 seconds.
8. Open the lid and remove the DEPC water chip.
9. Leave the lid open and wait about 60 seconds for any water remaining on the electrodes to evaporate.

E. PRIME THE CHIP

1. Open the Experion priming station by pressing down on the front lever.
2. Remove an Experion RNA StdSens chip out of its packaging and place it on the chip platform, matching the arrow on the chip with the alignment arrow on the chip platform. A post on the chip prevents insertion in the wrong position. Do not force the chip into position.
3. Pipet 9 µl filtered gel-stain solution (from Step 2.5) into the well labeled **GS** (gel priming well). Insert the tip of the pipet vertically and to the bottom of the well when dispensing. Do not expel air at the end of the pipetting step.



Warning: Placing the pipet tip at the edge of the well or allowing the gel to slide down the wall of the well may lead to bubble formation at the bottom of the well. It is acceptable to allow 1–2 small bubbles at the surface. Dislodge bubbles at the bottom with a clean pipet tip, or remove the gel-stain and refill the well.

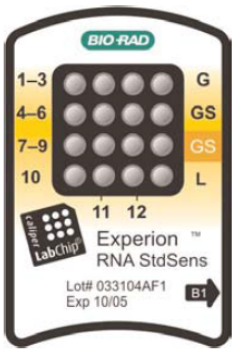
4. Carefully close the priming station by gently pressing down on lid. The lid should snap completely closed.
5. Set the pressure setting to **B** and the time setting to **1**, as specified by the alphanumeric code on the chip.
6. Press the **Start** button. The "Priming" message will illuminate on the LCD screen, the priming station will pressurize, and the timer will count down. Complete priming requires approximately 30 seconds. Do not open the priming station during the count-down.
7. An audible signal indicates that priming is complete, and a "Ready" message will be displayed. Open the priming station by pressing down on the release lever.
8. Turn the chip over and inspect the microchannels for bubbles or evidence of incomplete priming. The glass chip will appear opaque and the microchannels will be difficult to see if they are primed properly. If you detect a problem, such as a bubble or incomplete priming, prime a new chip.
9. Place the chip on a clean surface for loading samples.

After priming, check the back of the chip. The thin lines we could see before should be gone. Check there are no bubbles. If there is any problem, prime a new chip.

F. LOADING THE SAMPLES AND RNA LADDER INTO THE CHIP

Check Experion detailed instructions_biorad

1. Pipet 9 µl of the gel-stain solution into the other well labeled **GS**.
2. Pipet 9 µl of filtered gel into the well labeled **G**.
3. Pipet 5 µl of the loading buffer (yellow cap) in each sample well (1–12) and the ladder well, labeled **L**.
    - Use a new pipet tip for each delivery to prevent contamination of the loading buffer stock. Alternatively, remove 70 µl of loading buffer into an RNase-free tube and pipet 5 µl to each well from this volume
    - Make sure the pipet tip is centered and positioned vertically all the way to the bottom of the well. Avoid introducing bubbles into the bottom of the wells. It is acceptable to allow 1–2 small bubbles at the surface
    - All wells should be filled with the loading buffer, even when fewer than 12 samples are run. The chip will not run properly unless all wells are filled



4. Pipet 1 µl denatured RNA ladder into the well labeled **L**.
    - Every chip **must** have the RNA ladder loaded into the ladder well for accurate quantitation of samples and for sample alignment
5. Pipet 1 µl sample into each of the 12 sample wells.
6. If running fewer than 12 samples, add 1 µl loading buffer, TE buffer, or DEPC-treated water to the unused sample well(s).
7. Place the chip in the Experion vortex station.
8. Turn on the vortexer, which will operate for 60 seconds and then automatically shut off. Remove the chip when the vortexer stops.
9. Start the run immediately (within 5 minutes) to prevent excessive evaporation and poor results or a chip performance error.

G. RUN THE ANALYSIS

Place the primed chip with the samples in the electrophoresis station. Ensure that the chip is seated properly and then carefully close the lid.

Select **New Run**. Select the RNA **Std**Sens protocol_**Eukaryotic total RNA**.

Select **project** Sponge

Select **number of samples** to run (usually 12). Click the **Start** button to begin the chip run.

After a run has started, the green LED in the center of the front panel on the electrophoresis station will begin blinking.

If IV check fail occur → there are bubbles or an empty well. STOP the run and check the chip. Remove the bubbles or reload those wells if necessary then try to run it again.

When the chip run is complete, a "Run complete" message will be displayed. Remove the chip from the electrophoresis station and dispose of it. To prevent contamination of the electrodes, do not leave the chip in the electrophoresis station for an extended period of time. Also, it is a good practice to **immediately insert the DEPC water chip** (see next section) as soon as the RNA chip is removed to prevent samples and/or buffers from drying on the electrodes.

H. CLEAN THE ELECTRODES AFTER A RUN

1. Fill the cleaning chip labeled DEPC water with 800 µl DEPC-treated water. Gently tap the side of the cleaning chip to remove any trapped bubbles from the wells.
2. Open the lid of the electrophoresis station and place the cleaning chip on the chip platform.
3. Close the lid and leave it closed for about 60 seconds.
4. Open the lid and remove the DEPC water chip.
5. Allow the electrodes to dry for 30–60 seconds.
6. Close the lid.

Save the results in the pen-drive.

Analysis:

Take into account that the run settings are designed for eukaryotic total RNA. However, sponges samples (HMA) of total RNA have also important amount of prokaryotic RNA. So,instead of the typical 2 peaks of rRNA (18S and 28S) our samples will show 4 peaks: 16S, 18S, 23S and 28S. Because of this, sometimes the software mislabel the peaks. Eukaryotic 18S and 28SrRNA are in this case the 2nd and the 4th peak that appear in the graphs. If it is mislabeled, correct them before exporting the results.

10.Declaration of authorship

Erklärung zur Bachelorarbeit/Masterarbeit

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbstständig verfasst wurde und dass keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden. Diese Erklärung erstreckt sich auch auf in der Arbeit enthaltene Graphiken, Zeichnungen, Kartenskizzen und bildliche Darstellungen.

Bachelor's/Master’s thesis statement of originality

I hereby confirm that I have written the accompanying thesis by myself, without contributions from any sources other than those cited in the text and acknowledgements.
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