Osmoadjustment in the Coral Holobiont

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ABSTRACT

Coral reefs are under considerable decline. The framework builders in coral reefs are scleractinian corals, which comprise so-called holobionts, consisting of cnidarian host, algal symbionts (genus *Symbiodinium*), and other associated microbes. Corals are commonly considered stenohaline osmoconformers, possessing limited capability to adjust to salinity changes. However, corals differ in their ability to cope with different salinities. The underlying mechanisms have not yet been addressed. To further understand putative mechanisms involved, I examined coral holobiont osmoregulation conducting a range of experiments on the coral *Fungia granulosa*.

In my research *F. granulosa* from the Red Sea exhibited pronounced physiological reactions (decreased photosynthesis, cessation of calcification) upon short-term incubations (4 h) to high salinity (55). However, during a 29-day *in situ* salinity transect experiment, coral holobiont photosynthesis was unimpaired under high salinity (49) indicating acclimatization. *F. granulosa* microbiome changes after the 29-day high salinity exposure aligned with a bacterial community restructuring that putatively supports the coral salinity acclimatization (osmolyte synthesis, nutrient fixation/cycling). Long-term incubations (7 d) of cultured *Symbiodinium* exhibited cell growth even at ‘extreme’ salinity levels of 25 and 55. Metabolic profiles of four *Symbiodinium* strains exposed to increased (55) and decreased (25) salinities for 4 h indicated distinct carbohydrates and amino acids to be putatively involved in the osmoadjustment. Importantly, under high salinity the osmolyte floridoside was consistently increased. This could be corroborated in the coral model *Aiptasia* and in corals from the Persian/Arabian Gulf, where floridoside was also markedly increased upon short- (15 h) and long-term (>24 months) exposure to high salinity, confirming an important role of floridoside in the osmoadjustment of cnidarian holobionts.
This thesis demonstrates osmoacclimatization of *F. granulosa* and osmoadjustment of cultured *Symbiodinium*. All three main compartments (i.e. coral host, *Symbiodinium*, bacteria) seem to contribute to the coral holobionts salinity adjustment. However, the exact mechanisms of coral host and bacteria contribution remain to be determined. Floridoside likely constitutes a conserved osmolyte increasing the salinity resilience of *Symbiodinium* and also of the cnidarian/coral holobiont. Floridoside further possess’ antioxidative properties, possibly providing a protection from reactive oxygen species formation as a result of salinity stress or/and other environmental stressors.
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\(\Delta F\) \quad \text{\(Fm' – F\) (maximum light-adapted fluorescence - steady state fluorescence)}

\(\Phi_{\text{PSII}} = \Delta F / Fm'\) \quad \text{Effective quantum yield}

AMOVA \quad \text{Analysis of molecular variance}

ANOVA \quad \text{Analysis of variance}

BEST \quad \text{Statistical biological-environmental matching routine}

Brine \quad \text{Hypersaline discharge}

COO \quad \text{Compatible organic osmolyte}

DNA \quad \text{Deoxyribonucleic acid}

DMS \quad \text{Dimethyl sulfide}

DMSO \quad \text{Dimethyl sulfoxide}

DMSP \quad \text{Dimethylsulfoniopropionate}

DO \quad \text{Dissolved oxygen}

ENSO \quad \text{El Niño–Southern Oscillation}

ETC \quad \text{Electron transport chain}

\(F\) \quad \text{Steady state fluorescence}

FACS \quad \text{Fluorescence assisted cell-sorting}

\(Fm'\) \quad \text{maximum light-adapted fluorescence}

FSW \quad \text{Filtered seawater}

GC/MS \quad \text{Gas chromatography–mass spectrometry}

HBA \quad \text{Hydroxy benzylic acid}

NIST-MS \quad \text{National Institute of Standards and Technology Mass Spectral Library}

OTU \quad \text{Operational taxonomic unit}

PAG \quad \text{Persian/Arabian Gulf}

PAM \quad \text{Pulse amplitude modulated (fluorometer/fluorometry)}

PAR \quad \text{Photosynthetic active radiation [\(\mu\text{mol photons m}^{-2}\text{s}^{-1}\)]}

PBS \quad \text{Phosphate buffered saline}

PCoA \quad \text{Principal Coordinate Analysis}

PCR \quad \text{Polymerase chain reaction}

PHB \quad \text{Polyhydroxybutyrate}

PSII \quad \text{Photosystem II}

RNA \quad \text{Ribonucleic acid}

ROS \quad \text{Reactive oxygen species}

RT \quad \text{Retention time}

SIMPER \quad \text{Similarity percentage analysis}

SST \quad \text{Sea surface temperature}

SWRO \quad \text{Seawater reverse osmosis (desalination plant)}

TA \quad \text{Total alkalinity}

UDP \quad \text{Uridine diphosphate}

VLP \quad \text{Virus-like particles}
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1. INTRODUCTION

1.1. Coral reef ecosystems

Coral reefs form the largest biogenic structures, visible even from space. Estimates for global coral reef cover range between 255,000 km$^2$ and 1 million km$^2$ (excluding deep sea corals), covering approximately 0.1 - 0.5% of the ocean floor (Copper, 1994; Spalding and Grenfell 1997). Those reefs typically occur in tropical shallow waters, provide shelter for almost a third of all marine fish species, and about one third of all marine organisms are estimated to depend on coral reefs during their life cycle (McAllister, 1991; Reaka-Kudla et al., 1996). Harboring a multitude of micro- and macro-organisms coral reefs contain the largest biodiversity of all marine ecosystems and range amongst the most productive ecosystems on the planet (Connell, 1978; Moberg and Folke, 1999; Roberts et al., 2002). Beyond their importance as a biodiversity hotspot, coral reefs offer numerous goods and services, for example they support fisheries, coastline protection, and tourism industries (Moberg and Folke, 1999). 500 million people in more than 100 countries depend on coral reefs including at least 30 million directly reliant on protection for their livelihood or land they live on (Wilkinson, 2008). Consequently, their economic value is immense. Ecosystem benefits include estimates of US$ 375 billion and up to US$ 9 trillion per year (Costanza et al., 1997; de Groot et al., 2012) with a recent estimate of US$ 5.7 billion alone for the Great Barrier Reef (Hoegh-Guldberg, 2015). The keystone species or so-called framework-builders of coral reef systems are reef-building corals, which are responsible for the iconic 3D-structures of coral reefs.

1.2. The coral holobiont

For coral reefs, the order Scleractinia is of particular significance: It includes hard or stony corals, which are hermatypic, i.e. they secrete a calcium carbonate (CaCO$_3$) skeleton.
Scleractinian corals are primarily residing in the light-flooded zone of warm tropical and subtropical oceans (Kleypas et al., 1999). These habitats are commonly oligotrophic providing only limited nutrition. The key for the productivity of scleractinian corals is an obligate symbiosis with autotrophic dinoflagellates of the genus *Symbiodinium* (Gardiner, 1931; Muscatine and Porter, 1977). The coral host is not only associated to the unicellular, eukaryotic algae, but like all other multicellular organisms, also with a complex array of microorganisms (microbiome) (McFall-Ngai et al., 2013), which include Bacteria, Archaea, viruses, and Fungi among others (Rohwer et al., 2002; Rosenberg et al., 2007). The estimated density of coral microbes ranges between $1 \times 10^2$ and $1 \times 10^6$ cells per cm$^2$ coral tissue (Garren and Azam, 2012). Coral host, phototrophic algae, and the microbiome form a metaorganism - the coral holobiont – in which each compartment provides ‘goods and services’ for each other (Knowlton and Rohwer, 2003; Rosenberg et al., 2007).

The animal host from the phylum Cnidaria provides a sheltered environment, and metabolic products for its symbionts. The *Symbiodinium* cells, reside in the gastrodermal tissue of their coral host. They are wrapped in so-called symbiosomes, membrane-bound vacuoles that enable adjustment of favorable conditions (Trench, 1979; Wakefield and Kempf, 2001). The symbiotic dinoflagellates are provided with inorganic nutrients, carbon dioxide, and protection from UV radiation from the coral host – the essential requirements for productive photosynthesis (Muscatine and Porter, 1977; Falkowski et al., 1984; Muscatine et al., 1989). In turn *Symbiodinium* provide up to 95% of the required energy in form of photosynthates to their animal host; and thereby the base for corals to thrive in oligotrophic waters (Muscatine et al., 1981; Muscatine et al., 1984). *Symbiodinium* are not exclusively found in Scleractinians, but also for example in foraminifers, mollusks, sponges, or free-living (Carlos et al., 1999). The dinoflagellate genus includes an enormous diversity and has been divided into nine phylogenetic clades, A to I (Coffroth
and Santos, 2005; Pochon and Gates, 2010) and further into hundreds of different phylotypes (e.g. A1, A2, C1, etc.) (Baillie et al., 2000; LaJeunesse, 2001). However, phylotypes do not necessarily correspond with the species concept and further effort for a more universal species delineation are being undertaken (sensu Thornhill et al., 2014; Hume et al., 2016).

The exchange of ‘goods and services’ between coral and Symbiodinium is relatively well known, though the research addressing the function of other associated microbiota is still fragmentary (Bourne et al., 2016). The prokaryotic fraction includes mainly bacteria and has received increasing attention in the last two decades. According to the coral probiotic hypothesis, the microbiome is believed to aid the coral holobiont to adapt to environmental changes (Reshef et al., 2006). The microorganisms may further play a role in coral health, disease, and evolution (Rosenberg et al., 2007). The bacterial communities are highly diverse and complex, and can host thousands of unique operational taxonomic units (OTUs; a concept used synonymously for species) (Mouchka et al., 2010; Sunagawa et al., 2010; Blackall et al., 2015). The assemblages are different from their surrounding marine environment and sediments (Rohwer et al., 2002; Garren and Azam, 2012), and are oftentimes species-specific (Rohwer et al., 2002; Morrow et al., 2012). At the same time, they have also been shown to change seasonally (Koren and Rosenberg, 2006) and differ according to their life history stage (Sharp et al., 2012), geographic location (Klaus et al., 2005), environmental conditions (Roder et al., 2015), and location within the coral (Ainsworth et al., 2010). In this context, bacterial communities found in coral mucus, gastric cavity, coral tissue, and coral skeleton differ (Sweet et al., 2011).

Coral associated bacteria have been shown to be pathogenic, commensal, and mutualistic (Bourne and Webster, 2013). For example, Vibrio bacteria have been implicated in coral disease processes (Rosenberg and Falkovitz, 2004), and bacteria including members of
the genus *Endozoicomas* may be involved in pathogen defense (Glasl *et al.*, 2016). *Endozoicomas*, a bacterial genus commonly found associated with corals, has been located within the coral cells and suggested to be an endosymbiont involved in nutrient transport and cycling (Neave *et al.*, 2016). Other functions attributed to coral associated bacteria include microbial defense from pathogens (Ritchie, 2006; Reina *et al.*, 2016), nutrient acquisition (Lesser *et al.*, 2004), and nutrient cycling (Raina *et al.*, 2009; Kimes *et al.*, 2010; Rädecker *et al.*, 2015). Yet, assigning a specific role to the extremely diverse suite of bacteria remains difficult (Bourne and Webster, 2013; Bourne *et al.*, 2016).

Besides bacteria, Archaea are a constituent of the prokaryotic microbial communities. They play a minor role but are also thought to be involved in nutrient recycling (Siboni *et al.*, 2008; Bourne *et al.*, 2016). Literature on coral related Fungi mostly identified them as (potential) pathogens (Knowlton and Rohwer, 2003; Vega Thurber *et al.*, 2009; Moree *et al.*, 2013), but also showed involvement in nutrient cycling (Wegley *et al.*, 2007). The role of viruses or virus-like particles (VLP) is also unclear (Bettarel *et al.*, 2015), even though coral associated viruses and VLPs are receiving increased attention (Nguyen-Kim *et al.*, 2015; Weynberg *et al.*, 2015; Wood-Charlson *et al.*, 2015; Correa *et al.*, 2016; Vega-Thurber *et al.*, 2017).

To date, a multitude of coral associated microbial assemblages has been determined. However, the mechanisms establishing and controlling specific symbioses are mostly unknown and have become the interest of collaborative research (Voolstra *et al.*, 2015).

### 1.3. Threats for coral reefs

Dramatic changes in coral reef health have been reported from every part of the world. There is an ongoing worldwide decline of corals and between 50 and 70% of all coral reefs
are under direct threat (Hoegh-Guldberg, 1999; Gardner et al., 2003; Hughes et al., 2003; Bellwood et al., 2004; Carpenter et al., 2008). Commonly, coral reefs occur in relatively stable equatorial environments where they thrive in a narrow range of environmental parameters (Kleypas et al., 1999; Wood, 1999). In the last decades many of these parameters have been altered. Anthropogenic induced climate change is increasing CO$_2$ level in the atmosphere on a global scale. Higher CO$_2$ levels lead to global warming with increasing water temperatures, but also to a decrease in the oceans pH, a phenomenon called ocean acidification. Both may severely impact coral reef health on a global scale (Hoegh-Guldberg et al., 2007). Decreased pH is predicted to severely impact coral growth (Anthony et al., 2008). In comparison, elevated sea surface temperatures (SST) already impact coral health, growth, and reproduction (Hoegh-Guldberg, 1999). Coral mass die-offs linked to increased water temperature have been observed repeatedly in the last decades and are oftentimes accompanying El Niño Southern Oscillation (ENSO) events (Hoegh-Guldberg, 1999). SST anomalies resulting in coral mass bleaching events are predicted to further increase in frequency and severity (Hoegh-Guldberg, 1999; Hughes et al., 2003). In 2015/2016 the third global mass bleaching event caused by ENSO related increased SST has been the longest on record with dramatic coral bleaching and coral mortality recorded (Normile, 2016).

Coral bleaching is referred to as the dissociation of the coral-Symbiodinium symbiosis, during which the coral host loses large numbers of Symbiodinium cells and/or their photopigments leading to a loss of coloration (Hoegh-Guldberg, 1999). Besides temperature changes, coral bleaching can also be induced or facilitated by changes in UV radiation, nutrient level, and salinity (Lesser, 2011). On a cellular level, bleaching is believed to be triggered by oxidative stress (Lesser, 1997; Lesser, 2006). The environmental stressor generally causes a malfunction in photosystem II, leading to failure of the electron-photon
conversion and in the electron transport chain (ETC) (Lesser, 2006). The ETC disruption increases the formation of reactive oxygen species (ROS), which can further damage the photosynthetic apparatus, but also other cell compartments like cell organelles, proteins, enzymes, or even DNA (Lesser, 2011). The coral and its symbionts must counteract ROS formation, for example by producing antioxidants. Failure to produce sufficient antioxidant activity combined with ongoing high ROS levels can lead to a loss of the algal symbionts and coral bleaching (Downs et al., 2002). Generally corals can recover from bleaching, but depending on the magnitude and duration of the stress and on the particular coral species it may lead to mortality (Rodrigues and Grottoli, 2007).

On a more regional scale, climate change also leads to an increase in extreme weather events, including higher frequencies of storms, and changes in precipitation and evaporation patterns (Meehl et al., 2000; IPCC, 2014). Storms can physically devastate reefs and an increased (riverine) freshwater inflow may severely impact corals (Hughes, 1994; Tan et al., 2012). In addition to climate change related threats, other anthropogenic activities impact coral reef ecosystems. Eutrophication, pollution, and increased sedimentation from human engineered terrestrial environments, and over-exploitation of marine species, mining, and physical destruction by reef users are the main causes of local reef destruction (Sebens, 1984). Even though the impact of local disturbances to coral reefs seems minor in comparison to the global threats, a coral’s ability to recover from climate change related stress is significantly reduced if it is living in degraded water that contains pollutants (Negri et al., 2011). Environmental disturbances can alter the corals non-algal microbial community and function (Bourne et al., 2008; Bourne et al., 2009; Vega Thurber et al., 2009). Worldwide coral diseases have been linked to climate change and to degraded water quality, disrupting the coral holobiont homeostasis and thus facilitating opportunistic and/or pathogenic bacteria, viruses, or fungi (Harvell et al.,
Especially in the Caribbean, coral diseases were heavily impacting corals (Goldberg and Wilkinson, 2004; Harvell et al., 2007).

1.4. Coral acclimatization and adaptation

Environmental parameters determining coral distribution and growth include temperature, light intensity, salinity, nutrient level, and aragonite saturation state amongst others (Kleypas et al., 1999). Especially changes in parameters related to climate change (e.g. SST, pH, and salinity) are already affecting coral reef habitats or are predicted to do so within this century (Hughes et al., 2003; IPCC, 2014). To cope with the changes corals are forced to acclimatize and/or adapt or disappear and lead to species shifts towards more resilient/tolerant species.

Acclimatization of the coral holobiont is the physiological response of the organism to environmental stimuli that takes place within a lifetime. Adaptation are genotypical properties that are subject to selection and are passed on as hereditary traits. In other words, acclimatization incorporates the phenotypic plasticity of a genotype, whereas adaptation is a change of the genotype. Considering the holobiont framework, understanding acclimatization and adaptation requires consideration of all compartments as they specify each other. In this context, the hologenome theory defines metazoans and their associated microbes as biomolecular networks where the microbes have the potential to influence their hosts form, function, and fitness (Zilber-Rosenberg and Rosenberg, 2008; Bordenstein and Theis, 2015). For example, insect microbiota may influence mating preferences and/or frequency and may even influence speciation (Ezenwa et al., 2012; Lewis and Lizé, 2015; Brucker and Bordenstein, 2012). Resilience to
environmental changes like increased temperatures can also be strongly influenced by bacteria, as shown in aphids (Moran and Yun, 2015).

Accordingly, it has been indicated that microbiome and Symbiodinium contribute to coral holobiont acclimatization and adaptation. As in other long-lived sessile organisms, the coral host is considered susceptible to rapid environmental change (Hughes et al., 2003). In contrast, their unicellular associates, i.e. Symbiodinium and the microbiome, possess a much faster adaptation capacity based on their shorter generation times (Blackall et al., 2015). Importantly, the Symbiodinium and microbiome communities can adjust quickly by changes in the presence and/or abundance of specific members. The adaptive bleaching hypothesis (Buddemeier and Fautin, 1993) suggests that the exchange of the prevalent Symbiodinium type can enhance the holobionts resilience (Baker, 2001; Berkelmans and van Oppen, 2006; Jones et al., 2008). This is based on different Symbiodinium types differing in their acclimatization potential, e.g. in regard to temperature, irradiance, or nutrient availability (Brown, 1997; Gates and Edmunds, 1999). Therefore changes in the Symbiodinium composition may contribute to more productive host-symbiont combinations under different environmental conditions (Baker et al., 2013; Hume et al., 2015). Similar to Symbiodinium, the associated microbiome has been suggested to support the coral holobiont under changed environmental conditions (see coral probiotic hypothesis in 1.2.; (Reshef et al., 2006)). In support of this, microbiome changes have repeatedly been correlated with different environmental conditions (Roder et al., 2015; Hernandez-Agrida et al., 2016). However, a causal link showing improved fitness caused by microbiome members/composition is lacking owing to the complexity of the three compartment symbiosis.
1.5. Coral reefs in the Red Sea and the Persian/Arabian Gulf (PAG)

Corals thrive in a comparably narrow range of environmental parameters, especially in regard to temperature. Water temperatures exceeding their common upper thermal limit of about 30°C for an extended period by 1.0 – 1.5°C may lead to bleaching (Kleypas et al., 1999; Baker et al., 2008). However, the Red Sea and especially the Persian/Arabian Gulf (PAG) belong to the warmest water bodies that support coral growth with temperatures above 30°C (Fine et al., 2013; Hume et al., 2015; Roik et al., 2015; Roik et al., 2016). The Red Sea has a low freshwater inflow and limited water exchange with the Indian Ocean (Edwards and Head, 1987). It is generally oligotrophic with strong gradients of decreasing temperature and nutrients and increasing salinity (36 – 41) from North to South (Ngugi et al., 2012; Raitsos et al., 2013). The PAG provides even more challenging conditions for its corals. It is a shallow water body and has, similar to the Red Sea, limited freshwater inflow and water exchange with the Gulf of Oman (Coles, 2003). Consequently temperatures in summer reach above 36°C and below 12°C in winter, and salinity reaches regularly >45 (Kinsman, 1964; Sheppard et al., 1992). As in other regions, corals from the PAG and Red Sea are susceptible to ocean warming and bleaching events occur (Purkis and Rieg, 2005; Monroe et al., in revision). However, corals generally demonstrate some capability to acclimatize/adapt to increased temperatures (Coles and Brown, 2003; Palumbi et al., 2014; see also 1.4.). For example *Pocillopora verrucosa* has been shown to possess a high phenotypic plasticity and acclimatize along the temperature gradient of the Red Sea (Sawall et al., 2015). Further, Dixon et al., (2015) demonstrated the heritability of thermoresilience in *Acropora millepora* along the Great Barrier Reef. In this context Red Sea and PAG coral communities demonstrate that corals can survive and adapt to temperatures predicted for other habitats as a consequence of climate change.
Interestingly, the Red Sea and the PAG are not only the warmest water bodies that support coral growth but also the most saline with salinities up to 41 and above 49, respectively (Ngugi et al., 2012; D’Angelo et al., 2015). A connection between increased salinity and increased temperature resilience has been reported and suggests a mechanistic link (Coles and Jokiel, 1978; Porter et al., 1999).

1.6. Corals and salinity changes

Strong salinity changes are generally a rare scenario in coral reef environments. However, seasonal freshwater input, for instance caused by precipitation or riverine inflow in monsoonal regions, can lead to coral bleaching and mortality as a result of decreased salinity (hyposalinity) (Berkelmans et al., 2012; True, 2012). Corals may also at times be exposed to increased salinity (hypersalinity) in lagoons or pools at low tide. However, anthropogenic activities may lead to an increase of such scenarios. Generally, evaporation in low precipitation areas is predicted to increase and precipitation in high precipitation to increase resulting in lower and higher salinities, respectively (IPCC, 2014). Further, increased urbanization along the coastlines especially in arid and semi-arid regions will necessitate the constructions of desalination plants, which usually release high saline brine in the marine environment, including coral reef habitats (Lattemann & Höpner 2008).

To date, little is known on the potential consequences of salinity variations on scleractinian corals, especially considering predicted large scale changes (IPCC, 2014). Shick (1991) characterized sea anemones as osmoconformers, maintaining similar concentrations of osmolytes as the surrounding seawater. Similarly, corals are mostly considered stenohaline osmoconformers (Hoegh-Guldberg and Smith, 1989; Kerswell and
Jones, 2003; Hédouin et al., 2015), and already small changes in salinity have been demonstrated to result in serious impairment of coral functioning (Ferrier-Pages et al., 1999). Yet, some studies have also reported a resilience of certain species to salinity changes (Manzello and Lirman, 2003; Chartrand et al., 2009; Lirman and Manzello, 2009). Mayfield and Gates (2007) even suggested osmoregulatory mechanisms of the coral holobiont. In this context, anthozoans - including the coral animal - may have some ability to regulate their osmotic equilibrium, for instance by using free amino acids (dissolved in seawater or body fluids) as osmolytes (Shick, 1991; Mayfield and Gates, 2007). However, their algal symbionts produce carbohydrates like glycerol, which can be transported to the host (Muscatine, 1967). Glycerol is a common osmolyte, especially widespread in unicellular algae (Kirst, 1990). In marine algae and invertebrates both groups of osmolytes (i.e. free amino acids and carbohydrates) are used to counteract osmotic changes (Hellebust, 1976). In this context the symbiosis between coral host and Symbiodinium requires special consideration, as both systems cannot adjust their osmotic equilibrium independently from each other. For instance, a decrease in salinity may trigger the symbiont to adjust its internal osmotic pressure by releasing osmolytes. These are transferred to the host tissue, where they may further increase the osmotic misbalance between host and surrounding medium. On the other hand, upon increased salinities of the surrounding seawater Symbiodinium and coral host must both accumulate osmolytes or actively expel water.
1.7. References


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2. OBJECTIVES

This dissertation assesses the osmoregulatory capabilities of the coral holobiont. The present literature on osmoregulatory abilities of (scleractinian) corals includes studies reporting corals to be highly sensitive to salinity changes whereas others seem much more resilient. The global distribution of corals includes habitats with vastly different salinities, indicating corals to be generally able to thrive at different salinities. Yet, the physiological mechanisms involved in this acclimatization/adaptation have not yet been addressed, even though coral habitats are predicted to be affected from climate change related salinity variations. This lack of data was a major motivation for the herein presented research. In this work the Red Sea coral *Fungia granulosa*, cultured *Symbiodinium*, the coral model *Aiptasia*, and corals from the PAG and the Indopacific were exposed to salinity changes. The corals from the Red Sea and PAG presumably provide an exceptional opportunity to address their osmoregulatory capabilities considering their acclimatization/adaptation to salinities higher than in most other coral habitats (see 1.5.). In three chapters all compartments of the coral holobiont (i.e. animal host, algal symbiont, and associated bacterial community) were assessed for their putative response to changed salinities.

In brief, the first chapter assesses the photo-physiological response of the Red Sea coral *F. granulosa* exposed to long-term (29 d) increased salinity. The experimental set-up included an *in situ* coral transplantation experiment that employed high saline discharge from a seawater reverse osmosis (SWRO) desalination plant. It assesses the long-term effect of the high saline discharge on the photosynthetic efficiency of the coral *Fungia granulosa* along a transect. Further, physico-chemical parameters accompanying the discharge dilution are characterized. This chapter also aims to test for potential salinity
effects on the algal compartment by determining growth rates in cultured *Symbiodinium* exposed to long-term salinity changes.

The second chapter aims to disentangle temporal and compartment effects of the salinity response in *F. granulosa*. Here, in short-term incubation experiments the physiological short-term response of *F. granulosa* to increased salinities is assessed. This set-up enables not only to measure photosynthetic performance but also calcification rates of the coral host. Most importantly, this chapter characterizes changes in the associated microbial community of *F. granulosa* upon short- and long-term high salinity exposure and derives respective putative functional implications.

The third chapter focusses on the role of the algal symbiont. In high salinity short-term exposure experiments *Symbiodinium* cultures are screened for potential osmolytes and their role in the algal salinity acclimatization. Upon identification of a suitable candidate, its role is validated in the osmoregulation of different holobionts, including the coral model *Aiptasia* and different corals from the PAG and the IndoPacific.
CHAPTER I

3. **High salinity tolerance of the Red Sea coral *Fungia granulosa* under desalination concentrate discharge conditions: An *in situ* photophysiology experiment.**

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3.1. Abstract

Seawater reverse osmosis desalination concentrate may have chronic and/or acute impacts on the marine ecosystems in the near-field area of the discharge. Environmental impact of the desalination plant discharge is supposedly site- and volumetric-specific, and also depends on the salinity tolerance of the organisms inhabiting the water column in and around a discharge environment. Scientific studies that aim to understand possible impacts of elevated salinity levels are important to assess detrimental effects to organisms, especially for species with no mechanism of osmoregulation, e.g. presumably corals. Previous studies on corals indicate sensitivity towards hypo- and hyper-saline environments with small changes in salinity already affecting coral physiology.

To evaluate sensitivity of Red Sea corals to increased salinity levels, we conducted a long-term (29 days) in situ salinity tolerance transect study at an offshore seawater reverse osmosis discharge on the coral *Fungia granulosa*. While we measured a pronounced increase in salinity and temperature at the direct outlet of the discharge structure, effects were indistinguishable from the surrounding environment at a distance of 5 m. Interestingly, corals were not affected by varying salinity levels as indicated by measurement of the photosynthetic efficiency. Similarly, cultured coral symbionts of the genus *Symbiodinium* displayed high tolerance levels in regard to hypo- and hypersaline treatments.

Our data suggest that increased salinity and temperature levels from discharge outlets dissipate quickly in the surrounding environment. Furthermore, based on physiological state of photosystem II *Fungia granulosa* appear to tolerate levels of salinity that are distinctively higher than reported for other corals previously. It remains to be determined whether Red Sea corals in general display increased salinity tolerance, and whether this
is related to prevailing levels of high(er) salinity in the Red Sea in comparison to other oceans.

3.2. Introduction

A growing demand of freshwater in semi-arid and arid regions (e.g. Arabian Peninsula) leads to an increasing number of seawater desalination plants, especially the low energy consuming seawater reverse osmosis (SWRO) desalination plants (Fritzmann et al., 2007). As a result, more hypersaline concentrate discharge (brine) reaches the marine environments (Lattemann & Höpner 2008). Environmentally safe disposal of this brine is one of the key factors determining the environmental impacts of a desalination plant. The highest salinity that marine organisms can cope with in a desalination discharge area is defined as a salinity tolerance threshold. It depends on the species and the exposure time to elevated salinity levels (Voutchkov 2009).

Euryhaline marine organisms can commonly tolerate changes in salinity (in contrast to stenohaline species) and series of small increments are generally better tolerated than direct exposure to high salinities (Voutchkov 2009). Effects of concentrate discharges depend on exposure intensities, frequencies, the environment the brine is released into, and the brine temperature (Roberts et al., 2010). Accordingly, the effects of discharged brine can range from no significant impacts on microbial abundance or plankton communities, to widespread alterations in community structures of seagrass, invertebrates, soft-sediment infauna, and corals (Roberts et al., 2010 and references therein; van der Merwe et al., 2014).

Hermatypic corals are the key stone species of coral reefs, which are among the most diverse and productive ecosystems on this planet (Moberg & Folke 1999). Coral health
and survival fundamentally depend on the interaction between coral host and photosynthetic algae (genus *Symbiodinium*) that can be found in the endodermal tissues of reef-building corals. Unfortunately, research on the effects of brine discharge on corals is scarce. Mabrook (1994) reported corals disappearing from coastal areas in the Red Sea (Egypt) as a result of desalination plants discharge, but no reproducible data are presented. Corals are generally considered stenohaline osmoconformers and very sensitive to the effects of desalination plant discharge (Elimelech & Phillip 2011; Ferrier-Pages *et al.*, 1999; Manzello & Lirman 2003).

Besides a desalination discharge context, more data on coral salinity tolerance are available; especially for decreased salinities. Generally, changes in salinity may affect metabolism and/or photophysiology of the coral animal and/or the corals’ algal symbionts due to salinity stress (Chartrand *et al.*, 2009; Muscatine 1967). Moberg *et al.*, (1997) suggested that photosynthetic rates are lowered in proportion to the reductions in salinity (10 – 20), whereas respiration rates were either slightly decreased or unaltered for 2 hermatypic corals upon hyposaline treatments. Hoegh-Guldberg and Smith (1989) concluded moderately reduced salinity (30) exposure for 4-10 days at several temperatures does not induce bleaching in *Stylophora pistillata* and *Seriatopora hystrix*. At lower salinities (i.e. at 23) the authors reported death within 48 h. Only a limited number of studies have included the impacts of hypersalinity on corals (and the possible effect it might have on dinoflagellate symbiont functionality within the host). Generally, the effects of hyper- and hyposaline treatments are similar (Lirman & Manzello 2009; Muthiga & Szmant 1987). Severity of effects observed commonly coincides with salinity concentration, exposure time, coral species, and the speed of salinity change.
In this study, our objective was to determine long-term effects (29 days) of a strong salinity increase on the solitary coral *Fungia granulosa*. To do this, we transplanted corals along a 25 m transect from a SWRO facility discharge structure, and determined salinity, temperature, oxygen, and light levels regularly. At the same time, we measured the (photo) physiological state of the algal symbiont via PAM fluorometry and checked for signs of visual bleaching. PAM fluorometry quantifies the photosynthetic efficiency with which light energy is converted into chemical energy at photosystem II (PSII) level. Evaluating the chlorophyll fluorescence can indicate an organism’s photosynthetic efficiency under changing or stressful conditions, e.g. varying salinity regimes (Chartrand *et al.*, 2009), and therefore serves as a stress indicator. Additionally, we assessed the salinity tolerance of cultured *Symbiodinium* to understand the contribution of the algal symbiont to salinity tolerance of the coral holobiont.

3.3. **Material and Methods**

3.3.1. **Experiment overview**

The existing SWRO facility (i.e. the submerged discharge location) at the King Abdullah University of Science and Technology (KAUST) was selected for this case study. The plant is located on the KAUST campus and is designed to provide all potable water needs. Under current operational conditions, the raw water intake is about 2,825 m$^3$ h$^{-1}$ with a recovery rate of 39 %, resulting in an average brine flow of 1,723.25 m$^3$ h$^{-1}$ (41,358 m$^3$ d$^{-1}$) that is discharged to the Red Sea. The submerged outfall (discharge structure) is located at a water depth of 18 m, approximately 2.8 km from the pump station (22° 17.780N, 39° 04.444E). The concentrate is pumped through a 1.2 m diameter pipeline to the offshore structure where the concentrate is pushed up in a concrete riser and discharged
horizontally through four discharge screens (1,800 mm × 1,000 mm) approximately 6 m above the seafloor.

3.3.2. Experimental setup

We conducted an in situ salinity stress experiment on the coral *Fungia granulosa* collected from Fsar reef (22° 13.945N, 39° 01.783E, approximately 9 km from the study site) over 29 days (15.01.14 – 13.2.14). At Fsar Reef salinity, light conditions, and effective quantum yields for *Fungia granulosa* were measured for reference purposes. Corals were handled with latex gloves and 18 specimens were collected from 16 to 19 m (similar depth to experimental study area) into separate zip lock bags. Corals were transported to the study site in shaded opac plastic boxes filled with ambient sea water. All specimens were placed on the roof of the discharge structure (Figure 1), labeled with nylon fishing line and underwater paper tags, and left for acclimatization for 20 hours. Three specimens were then randomly selected and placed at each of the 6 stations at the discharge screen (station 1) and along a 25 m transect (stations 2 – 6) (Figure 2a, 2b).

![Discharge structure and stations assayed in this experiment.](image)
3.3.3. Data collection

We collected data on temperature, dissolved oxygen (DO), and salinity. For each sampling time point (T0 – T6) we sampled between 11:00h and 12:00h. We measured the effective quantum yield (ΔF/Fm') and visually assessed all specimens for signs of bleaching. Temperature was logged continuously in 10 min intervals with HOBO Pendant® temperature data loggers at each station over the entire experiment. For DO and salinity measurements, water samples were collected during each dive from each station. Water samples for salinity measurements were collected using 50 mL Falcon Conical Centrifuge Tubes and 1 L low-density polyethylene (LDPE) cubitainer were used for DO samples. Salinity and DO were measured for all stations immediately after each dive. DO measurements were conducted using a WTW (Multi) 3500i Multi-Parameter Water Quality Meter with a CellOx® 325 DO electrode and salinity with a WTW Cond 3310 Meter with TetraCon® 325. Salinity and DO were analyzed for significant differences via one-way ANOVA using Statistica 10 (StatSoft Inc. 2011, version 10). A diving PAM fluorometer
(DIVING-PAM, Walz, Germany) was used to measure photosynthetically active radiation (PAR) [μmol photons m$^{-2}$ s$^{-1}$] at each station and to evaluate the effective quantum yields ($\Delta F/Fm'$) of the symbiotic algae of each coral specimen. The effective quantum yield ($\Delta F/Fm'$) of photochemical energy conversion in PSII for each measurement was calculated based on $F$ and $Fm'$ according to:

$$\Phi_{PSII} = (Fm' - F)/Fm' = \Delta F/Fm'$$  
(Genty et al., 1989).

$F$ and $Fm'$ measurements for each specimen were conducted between 11:00h and 12:00h to ensure comparable daytime conditions (in regard to physiology and prevailing light regime). All $\Delta F/Fm'$ measurements were taken in triplicate for each coral and specimens were only collected after the last sampling event (29 days).

### 3.3.4. Symbiodinium culturing and salt gradient stress experiment

*Symbiodinium microadriaticum* CCMP2467 (clade A1) was cultured in Guillard and Ryther F/2 media suspension without silicium in an incubator at a temperature of 26 °C and with a light intensity set at 4 μmol photons m$^{-2}$ s$^{-1}$ (as measured by diving-PAM) (Guillard & Ryther 1962). The F/2 media was newly prepared from sea water (obtained from 100 m depth in the Red Sea) and completed with 0.5 ml NaNO$_3$, NaH$_2$PO$_4$, vitamins and trace metals following Guillard and Ryther (1962). The cells were kept in the exponential growth phase with a cell density between $10^5$ to $10^6$ cells mL$^{-1}$ for two weeks prior to experiments. For the comparability of experiments, the cells were then directly transferred into a freshly prepared salt adjusted F/2 media with a cell density of ~1x10$^5$ cells mL$^{-1}$. A salt gradient experiment was conducted in concentrations ranging from 25 to 55 (in increments of 5). The F/2 media were prepared by adding appropriate amounts of double-distilled water (ddH$_2$O) for a diluted salinity range of 25 – 35. In order to obtain
elevated salinity levels on the order of 45 – 55, the media was spiked with NaCl (Sigma). Cells were then transferred into sterilized plastic culture flasks in equal volumes (400 mL) of adjusted F/2 media to reach a cell density of ~1x10^5 cells mL^−1. Cells were sampled each day for a seven day period (0 d, 1 d, 2 d, 3 d, 5 d, and 7 d) at a temperature of 28 °C and under a 9 μmol photons m^−2 s^−1. The 0 d sample was used as a control and only withdrawn from cells at the 40 (ambient) condition. Sample volumes for fluorescence activated cell sorting (FACS) analysis were 1 mL, which were directly harvested by centrifugation (5430 R centrifuge, Eppendorf) at 10’000×g for 10 min at 4 °C. For cell fixation, 700 μL media were withdrawn, adding 100 μL of 40 % formaldehyde in ddH2O to each of the samples to reach a final concentration of 10 %. Cells were thoroughly resuspended by vortexing and kept at 4 °C until further analysis. Following cell fixation, *Symbiodinium* cells were again harvested by centrifugation, supernatant discarded, and washed once with 500 μL buffer solution (*phosphate buffered saline* (PBS)). PBS was removed completely and 500 μL SYBR Green DNA (2x conc., Life Technologies) staining in PBS was added. Cells were resuspended by vortexing and stained for 1h at room temperature at 400 rpm in a ThermoMixer® (Eppendorf). After staining, cells were again pelleted by centrifugation, washed once with PBS and finally resuspended in 1 mL PBS. For FACS measurements 200 μL of each sample were transferred into a 96 well flat bottom well plate and measured. FACS measurements were conducted on a BD LSRFortessa™ cell analyzer (BD Bioscience, US) using the 405 nm violet laser and QDot655 filters for chlorophyll fluorescence. SYBR Green fluorescence was excited via the 488 nm blue laser and emission detected via *Alexa Fluor® 488* filters for total nucleotide detection. FACS data was analyzed by FlowJo 7.5 flow cytometry analysis software.
3.4. Results

3.4.1. Ecological conditions

Water temperature along the transect showed a range between 24.26 °C and 28.46 °C (Table 1). Average water temperatures recorded at the discharge screen (26.3 °C ± 0.78 °C) were higher than at the other stations (25.83 °C - 26.08 °C). Average water temperatures also decreased with increasing distance from the discharge structure. DO varied between 5.75 mg L⁻¹ and 6.37 mg L⁻¹ with average DO levels being 6.07 ± 0.17 mg L⁻¹ and no significant differences in DO observed between the stations (PANOVA ≥ 0.05). In contrast to DO, salinity data showed significant differences (Figure 3). At the discharge screen (station 1) salinity differed significantly from the other stations (PANOVA ≤ 0.05). We found no significant difference between all other stations (PANOVA ≥ 0.05) with an average of 41.3 ± 0.7. However, salinity decreased slightly with increasing distance from the discharge (Figure 3). Control measurements at Fsar reef (site of coral collection) showed a salinity of 39.

Table 1. Temperature data (HOBO Pendant® Temperature Data Loggers) from all transect stations. Logging interval 10 min; temperature [°C] mean ± SD; minimal and maximal measured temperature.

<table>
<thead>
<tr>
<th></th>
<th>Station 1 (Discharge)</th>
<th>Station 2 (0 m)</th>
<th>Station 3 (2.5 m)</th>
<th>Station 4 (5 m)</th>
<th>Station 5 (15 m)</th>
<th>Station 6 (25 m)</th>
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<tr>
<td>AVG</td>
<td>26.30</td>
<td>26.08</td>
<td>26.02</td>
<td>25.99</td>
<td>25.84</td>
<td>25.83</td>
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<tr>
<td>± SD</td>
<td>± 0.78</td>
<td>± 0.63</td>
<td>± 0.62</td>
<td>± 0.64</td>
<td>± 0.73</td>
<td>± 0.95</td>
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<tr>
<td>Min</td>
<td>24.74</td>
<td>24.64</td>
<td>24.55</td>
<td>24.45</td>
<td>24.35</td>
<td>24.26</td>
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<tr>
<td>Max</td>
<td>28.46</td>
<td>27.76</td>
<td>27.67</td>
<td>27.57</td>
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</table>
Figure 3. Salinity concentrations measured for each sampling event at each station (6 time points, 6 stations). Average salinities: 49.4 ± 2.0 (discharge screen), 41.8 ± 0.3 (0 m), 41.3 ± 0.6 (2.5 m), 41.3 ± 1.0 (5 m), 41.2 ± 0.6 (15 m) and 41.0 ± 0.7 (25 m), respectively. AVG shows the average salinity at each station.

3.4.2. Coral salinity tolerance

The effective quantum yield ($\Delta F/Fm'$) was measured for all 18 coral colonies in triplicates for all time points (i.e. T0 – T6) (Figure 4). Control measurements from corals in their natural reef environment showed average $\Delta F/Fm'$ of 0.700 ± 0.010 (at 30 - 50 μmol photons m$^{-2}$ s$^{-1}$; triplicate measurements on three specimens). $\Delta F/Fm'$ at all stations were constant and in the same range as the controls. Average reads were 0.705 ± 0.009 (station 1), 0.694 ± 0.008 (station 2), 0.687 ± 0.007 (station 3), 0.687 ± 0.019 (station 4), 0.693 ± 0.017 (station 5), and 0.690 ± 0.02 (station 6). We found a drop in $\Delta F/Fm'$ at T1 for stations 4 – 6 which corresponds with elevated light conditions on this day compared to the other stations and sampling events (65 – 82 μmol photons m$^{-2}$ s$^{-1}$; Figure 4d, e, f). PAR levels ranged between 4 and 82 μmol photons m$^{-2}$ s$^{-1}$, with average reads of 14 ± 4
(station 1), 17 ± 5 (station 2), 33 ± 13 (station 3), 45 ± 27 (station 4), 34 ± 27 (station 5), and 28 ± 21 μmol photons m⁻² s⁻¹ (station 6). By trend, PAR levels at station 1 and 2 and to a smaller extent at station 3 where lower and more stable compared to the other stations (Figure 4). This pattern is caused by a shading effect of the discharge structure which kept light levels in its direct surrounding lower and more stable. No PAR was measured at time point T5. During the study we did not observe bleaching characteristics on the measured specimens.

Figure 4. Effective PSII quantum yields (\(\Delta F/F_m'\)) of individual *Fungia granulosa* colonies during the 29-day transplantation experiment at each station (a-f). Bars show photosynthetic active radiation (PAR).
3.4.3. Coral symbiont salinity tolerance

To confirm our in vivo observations, i.e. the absence of measurable detrimental effects of high salinity on the (photo) physiology of Fungia granulosa, we investigated the response of cultured coral symbionts, i.e. Symbiodinium microadriaticum, to a range of salinity levels. Chlorophyll a levels and growth rate of S. microadriaticum were investigated at salinities between 25 to 55 and samples were taken at seven time points (0 d, 1 d, 2 d, 3 d, 5 d, and 7 d). FACS cell counts and chlorophyll a level measurements showed that Symbiodinium cells reached the exponential growth phase after four days of incubation at salinities between 30 and 50 (Figure 5). The quickest cell proliferation was observed at 35. For concentration levels of 25 and 55, respectively, the cell growth rates were inhibited. Measuring chlorophyll levels by FACS showed no differences (data not shown).

Figure 5. Coral symbiont cell growth determined by FACS after incubation in salt adjusted F/2 media at salinity levels ranging from 25 – 55 (incubated at 28 °C, under a light intensity of 9 μmol photons m$^{-2}$ s$^{-1}$).
3.5. Discussion

In this study we (1) characterized physicochemical conditions along a 25 m transect at a SWRO facility discharge structure in the Red Sea. We measured (2) photosynthetic characteristics of *Fungia granulosa* as a response to a sudden and strong increase and continued elevated levels in salinity. We also checked for (3) indications of bleaching for the coral specimens exposed to the highest salinity levels directly at the discharge screen. Last, we (4) exposed *Symbiodinium* cultures to low and high levels of salinity to test salinity tolerance of the coral symbiont. With regard to the physicochemical conditions, we measured a decrease in salinity and temperature with increasing distance from the discharge structure. Of note, we already measured distinct lower values at stations 2 and 3 (seafloor, 0 and 2.5 m) compared to station 1 (discharge screen). This indicates a quick natural mixing of the brine in the study area to the point of showing little to no discernible salinity abnormalities (within short distances). These findings are in line with the literature (Roberts et al., 2010). Light levels were dependent on water clarity and measured light levels were in a similar range to what we measured at the collection site (Fsar reef, 30 – 50 μmol photons m$^{-2}$ s$^{-1}$). Stations 1 – 3 were shaded by the discharge structure and showed lower and more stable PAR levels.

These light patterns were also reflected in our $\Delta F/Fm'$ measurements, i.e. the photosynthetic characteristics of *Fungia granulosa* in response to changes in salinity (Figure 6). Measurements especially at station 1 and 2 (but also station 3) were more stable; in contrast stations 4 – 6 displayed stronger variations with a drop after 1 day (T1). This drop corresponds to noticeable clearer water and accordingly higher PAR values for the unshaded stations 4 – 6. All results are on the order of the 'baseline' yields measured under natural conditions considering variations in light intensity and suggest no discernable effect on PS II level of the dinoflagellate symbionts of the corals. On average
ΔF/Fm’ measures recorded at station 1 were higher than at the other stations (0.705 μmol photons m⁻² s⁻¹ compared to 0.687 – 0.694 μmol photons m⁻² s⁻¹). This again corresponds with lower average PAR values (14 μmol photons m⁻² s⁻¹ compared to 17 – 45 μmol photons m⁻² s⁻¹), but also underlines that the significantly higher salinity at station 1 does not negatively impact the photosynthetic efficiency of the coral’s symbionts. However, effective quantum yield measurements, reflecting (chronic) photoinhibition, may have limitations. Measurements from bleached corals may result in apparent healthy PAM yields, rather depending on the physiological state of associated Symbiodinium and less on the symbiont density or total number of cells (Fitt et al., 2001). Bleaching may exhibit a loss of Symbiodinium and/or pigments and can indicate a breakdown in the essential symbiotic relationship between coral host and algal symbiont (Brown 1997). Taking this into account, we visually inspected the corals during each sampling dive. We could not detect any apparent colony changes or loss in coloration (i.e. pigment loss) and thus exclude bleaching as a consequence of increased salinity during the duration of the experiment.
Our results demonstrate a high salinity tolerance of the Red Sea coral *Fungia granulosa*. In contrast, other studies observed substantial loss of pigmentation and/or symbionts at considerably smaller salinity changes. For example, Ferrier-Pages *et al.*, (1999) used a Red Sea clone of *Stylophora pistillata* that has been maintained in aquaria for several months. At moderately increased salinity levels (+2), coral colonies showed significant effects on photosynthesis, respiration, and protein content. Interestingly, the increased salinity of 40 corresponds with the salinity in *S. pistillata*’s natural habitat. In contrast, Lirman and Manzello (2009) found notable tolerance to salinity in *Siderastrea radians*, collected from Biscayne Bay, Florida where the authors measured highly variable levels of salinity. The authors also reported on *Porites furcata* (collected from the same bay) to be highly salinity tolerant (Manzello & Lirman 2003). In a study from Chartrand *et al.*, (2009), *Siderastrea*...
radians was collected from several different sites with different levels of natural salinity variations. The authors found a correlative trend of local salinity regimes and hyposaline stress tolerance levels. Colonies originating from less stable environments showed higher photosynthetic efficiency in hyposaline treatments than corals from more stable surroundings. These observations are in line with a recently conducted study, were Barshis et al., (2013) found ‘front-loading’ of genes to confer higher temperature tolerance levels in corals exposed to temperature-variable environments in comparison to cooler, stable environments.

To date, previous studies have mostly assumed corals to be stenohaline osmoconformers (Ferrier-Pages et al., 1999; Hoegh-Guldberg & Smith 1989; Kerswell & Jones 2003). In contrast, Chartrand et al., (2009) stated that a threshold response is indicative of the coral maintaining and successfully regulating its internal osmotic balance, which would contradict corals to be stenohaline osmoconformers. This is supported by a broad range of salinity tolerance levels found in different coral species depending on their original environment, which also indicates differently effective osmotic regulation. Furthermore, Mayfield and Gates (2007) discuss potential mechanisms involved in corals maintaining their osmotic balance. Accordingly, osmoregulatory processes presumably play a role in the performance of Fungia granulosa in this experiment. Osmoregulatory processes might be reflected by an initial reaction to the sudden salinity increase. This period presumably happened in our experiment between T0 and T1 and could be addressed by short-term studies. Since ambient salinity levels in the Red Sea are higher than in most other oceans (Douabul & Haddad 1970) salinity tolerance in Red Sea corals might generally be higher than for corals in other oceans.
Photophysiological resilience of *Fungia granulosa* towards high salinity is supported by our *Symbiodinium* culture study. Inhibited cell growth at extreme changes in salinity (i.e. 25 and 55) might be caused by limits in the cellular salinity regulation capabilities of *Symbiodinium* cells, namely Na\(^+\) pumps (Na\(^+\)-ATPase) (Goiran *et al.*, 1997). Similar to plants, high NaCl exposure levels could lead to degradation of chlorophyll a as observed in sunflower leaves (Santos 2004). Continuously lowered levels of active chlorophyll might result in a reduced energy uptake via photosynthesis and therefore also directly influence cell growth. However, salinity levels up to 50 (at par to highest measured *in situ* salinity levels) did not seem to inhibit cell growth in coral symbionts in culture. This demonstrates a wide plasticity (30 – 50) of *Symbiodinium* in regard to salinity changes, which has been found for hyposaline treatments previously (Chartrand *et al.*, 2009). This resilience furthermore indicates that the algal symbionts may generally not be determining the acclimatization potential of the coral holobiont towards salinity changes.

In conclusion, we found a quick mixing of discharged brine with surrounding waters based on salinity and temperature measurements. The photophysiology of the coral *Fungia granulosa* exposed to the discharge environment along a 25 m transect was not influenced by rapid and prolonged changes in salinity (but varied according to changes in light conditions). Our data characterize *Fungia granulosa* coral holobionts to be remarkably resilient towards increased salinity levels, which are potentially brought about by acclimatization to increased salinity levels in the Red Sea environment. Additionally, we showed that cell cultures of *Symbiodinium* only displayed inhibited cell growth at very high and low salinity levels. Based on our data we suggest *Fungia granulosa* from the Red Sea to possess high acclimatization potential to salinity changes, also in regard to future ocean scenarios.
3.6. **Author contributions & Acknowledgements**

TR, CRV, RVDM, MO designed and conceived the experiments. RVDM, TR, MO generated data. RVDM, TR, CRV, MO analyzed and interpreted data. SL, GA contributed reagents/materials/analysis tools. RVDM, TR, CRV, MO wrote the manuscript.

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3.7. **References**


CHAPTER II

4. Long-term salinity tolerance is accompanied by major restructuring of the coral bacterial microbiome

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4.1. Abstract

Scleractinian corals are assumed to be stenohaline osmoconformers, although they are frequently subjected to variations in seawater salinity due to precipitation, freshwater run-off, and other processes. Observed responses to altered salinity levels include differences in photosynthetic performance, respiration, and increased bleaching, and mortality of the coral host and its algal symbiont, but a study looking at bacterial community changes is lacking. Here we exposed the coral *Fungia granulosa* to strongly increased salinity levels in short- and long-term experiments to disentangle temporal and compartment effects of the coral holobiont (i.e. coral host, symbiotic algae, and associated bacteria). Our results show a significant reduction in calcification and photosynthesis, but a stable microbiome after short-term exposure to high salinity levels. By comparison, long-term exposure yielded unchanged photosynthesis levels and visually healthy coral colonies indicating long-term acclimatization to high salinity levels that were accompanied by a major coral microbiome restructuring. Importantly, a bacterium in the family *Rhodobacteraceae* was succeeded by *Pseudomonas veronii* as the numerically most abundant taxon. Further, taxonomy-based functional profiling indicates a shift in the bacterial community towards increased osmolyte production, sulfur oxidation, and nitrogen fixation. Our study highlights that bacterial community composition in corals can change within days to weeks under altered environmental conditions, where shifts in the microbiome may enable adjustment of the coral to a more advantageous holobiont composition.
4.2. Introduction

Coral reefs are among the most diverse and productive ecosystems on the planet (Reaka-Kudla et al., 1996) and provide a wide range of goods and services to approximately 500 million people in more than 100 countries (Wilkinson 2008). Coral reef ecosystems rely on the three-dimensional carbon skeleton framework built by scleractinian corals. Scleractinian corals are metaorganisms - so-called coral holobionts – that are composed of the coral host, its dinoflagellate endosymbionts (genus Symbiodinium), and a diverse microbial assemblage consisting of fungi, bacteria, archaea, and viruses (microbiome) (Rohwer et al., 2002). The bacterial microbiome has been shown to play important roles in coral health (Rosenberg et al., 2007), immunity (Ritchie 2011), as well as carbon, sulphur, and nitrogen cycling (Lema et al., 2012; Raina et al., 2009; Rohwer et al., 2002). Moreover, it has been suggested that microbial assemblages facilitate the acclimatization of coral holobionts to new environmental conditions (the probiotic hypothesis, Reshef et al., 2006). However, knowledge about the specific role of the vast majority of microbes, and in particular bacteria, to holobiont function is still limited (Barott et al., 2011; Bourne & Webster 2013; Lesser et al., 2004; Morrow et al., 2012).

Although frequently exposed to changes in seawater salinity, e.g. due to precipitation, freshwater runoff, periods of prolonged drought, or desalination processes (Chartrand et al., 2009; Edge et al., 2013; Hédouin et al., 2015; Lirman & Manzello 2009; Roberts et al., 2010), scleractinian corals are assumed to be stenohaline osmoconformers with a limited ability to adjust to salinity fluctuations (Ferrier-Pages et al., 1999; Hédouin et al., 2015; Hoegh-Guldberg & Smith 1989; Kerswell & Jones 2003; True 2012). Nevertheless, studies show that some coral species are able to tolerate greater salinity fluctuations than others. For instance, Stylotpora pistillata showed decreased respiration and photosynthetic rates at minor salinity decreases; whereas Siderastrea radians displayed a high resilience
towards salinity changes (Ferrier-Pages et al., 1999; Lirman & Manzello 2009). Further, Coles (2003) reported high salinity tolerance of corals in the Arabian Gulf and Red Sea, with some species surviving salinities of 48-50. While higher animals possess excretory systems to adjust for changes in salinity, most marine invertebrates, including scleractinian corals, are considered osmoconformers (Evans 2008; Yancey et al., 2002). Yet, in sea anemones changes in free amino acid pools are involved in osmoregulation (Shick 1991). Similarly, marine microorganisms (i.e. algae and bacteria) accumulate diverse molecules that serve as osmolytes upon increased salinity exposure (Csonka & Hanson 1991; Mayfield & Gates 2007). However, osmoregulation in the coral-algal endosymbiosis represents a challenging scenario. The coral animal host has to equilibrate the external osmotic pressure with its intracellular environment, which is determined by its own metabolism and that of its algal symbionts (Mayfield & Gates 2007). In this context the role of the coral-associated bacteria is virtually unknown.

To elucidate the role of coral-associated bacteria to salinity changes, we exposed the coral *Fungia granulosa* to strongly increased salinities resulting from seawater reverse osmosis (SWRO) desalination concentrate. By collecting data from all holobiont compartments (i.e. coral host, symbiont algae, bacterial microbiome) using a combination of short- (4 h) and long-term (29 d) experimental treatments, we aimed to disentangle compartment-specific responses and to assess potential adaptation/acclimatization processes by characterizing the initial response and long-term effects on the coral holobiont.
4.3. Material and Methods

4.3.1. Study site

We used concentrated salt brine from a seawater reverse osmosis (SWRO) desalination plant located at King Abdullah University of Science and Technology (KAUST, Saudi Arabia) to test the effects of increased salinity to *Fungia granulosa*. The SWRO currently discharges an average concentrated salt brine of 41,358 m$^3$ day$^{-1}$. The submerged discharge structure is located at 22°17.780N, 39°04.444E at 18 m depth with four discharge screens (1,800 × 1,000 mm) about 6 m above the seafloor (Figure 1) (van der Merwe et al., 2014b).

4.3.2. Short-term hypersalinity treatment

Hypersaline water with a salinity of 55 was collected directly from the desalination plant discharge. A hose was used to collect the concentrate via SCUBA and pumped into a container on an accompanying boat. The collected water was then transferred into 50 L opaque plastic bins and salinity was determined (WTW Cond 3310, WTW, Weilheim, GER). Ambient water (39) was collected from the top of the desalination discharge structure. We collected 14 specimens of *Fungia granulosa* (6-8 cm) on the same day (March 2014) in about 8 km distance at Fsar reef (22°13.945N, 39°01.783E; 14-18 m). The corals were sampled separately in zip-lock bags and transferred to 50 L opaque plastic containers filled with ambient reef water (39) upon return to the boat. Four specimens were immediately rinsed with filtered seawater (FSW; 0.22 µm), wrapped in aluminum foil, and flash frozen in liquid nitrogen for further analyses (freshly collected corals). The remaining ten coral specimens were photo-acclimated to constant experimental light conditions of 120 µmol m$^{-2}$ s$^{-1}$ (measured with DIVING-PAM, Walz, Effeltrich, GER) for 30 min at 27°C. Each coral specimen was measured thrice via pulse-
amplitude modulated fluorometry (PAM; measured with DIVING-PAM) for photochemical efficiency. Subsequently, ten specimens were transferred into one 1 L glass beaker each: five were placed in a 39 salinity ‘control’ bin and five in a 55 ‘high salinity’ bin, each holding 50 L of water. Four 50 mL water samples were taken from the control and treatment bin each over a 0.45 µm filter attached to a syringe for total alkalinity (TA) measurements. Oxygen concentration (WTW Multi 3500i, WTW), salinity, and temperature (both WTW Cond 3310) were measured within each of the coral beakers prior to incubation start. Incubations were stopped after 4 h beaker by beaker. Again, oxygen and photochemical efficiency were measured, and 50 mL water samples from each beaker for TA measurements were taken in duplicate. Corals were rinsed with FSW, wrapped in aluminum foil, and frozen in liquid nitrogen until further analysis.

4.3.3. Long-term hypersalinity treatment

We selected the KAUST SWRO discharge site (see above) for an in situ transplantation experiment that has been previously described in van der Merwe et al., (2014b). Previous measurements suggested increased salinities also at a greater distance (>15 m) from the discharge structure (van der Merwe et al., 2014a). To cover a range of salinities resulting from the brine discharge, and to assess the discharge dilution pattern, and the salinity impact on the coral *F. granulosa*, six stations were chosen along a transect: station 1 in the discharge screen, all other stations on the seafloor at 0, 2.5, 5, 15, and 25 m distance northwards from the discharge structure (Figure 1). Environmental parameters (i.e. salinity, temperature, dissolved oxygen, photosynthetically active radiation (PAR)) along the transect were assessed at each station (van der Merwe et al., 2014b). For the long-term experiment, 18 specimens of *F. granulosa* were collected from 14 to 18 m depth at
Fsar reef (January 2014). Collected corals were acclimated at ambient salinity on the desalination discharge structure roof for 20 h and tagged with nylon fishing line and labels. Three corals were randomly distributed to each of the six stations. The corals were fixed onto bricks (stations 2-6) and to the screen grid (station 1), respectively (van der Merwe et al., 2014b). All corals were measured thrice via PAM 1 h after attachment and visually assessed for any signs of bleaching; additionally water samples (1 L cubitainer) and PAR measurements were taken at each station. To test for the degree of correlation between changes in ambient light levels and effective quantum yield, we used SigmaPlot 11 (SYSTAT Software, Point Richmond, CA, USA) to conduct a linear regression analysis. As described in detail in (3.3), all water samples were measured for dissolved oxygen and salinity immediately upon return to the boat. The sampling procedure was repeated after 1, 4, 6, 8, 15, and 29 days and routinely took place between 11:00h and 12:00h. After 29 days water samples from each station were taken for assessment of reef water bacterial community. All coral specimens were collected into separate zip-lock bags, rinsed with FSW on board, wrapped in aluminum foil, and flash frozen in liquid nitrogen until further analysis.

4.3.4. Coral physiology
We determined coral calcification rate (G) according to the total alkalinity (TA) anomaly method (Schneider & Erez 2006). Each 50 mL water sample was analyzed for total alkalinity (TA) using an automated titrator (Titrando 888, Metrohm AG, Herisau, CH) with 0.01 M HCl. Following the Gran approximation, we used the second endpoint of the titration curve to estimate TA (Grasshoff et al., 2009). We used the difference in TA
between initial and final sample, normalized over incubation time (T) and coral tissue surface area (SA) to calculate G as follows (Schneider & Erez 2006):

\[
G \left[ \mu\text{mol CaCO}_3/\text{cm}^2/\text{h}^1 \right] = \frac{\Delta TA}{2} \times \frac{(V_{\text{jar}} - V_{\text{coral}} [L]) \times \text{water density [kg L}^{-1}]}{T \times \text{SA} \times \text{cm}^2}
\]

We used the difference of dissolved oxygen concentration at incubation start and end, and normalized over T and SA to calculate net photosynthesis \((P_n)\) (Schneider & Erez 2006):

\[
P_n \left[ \mu\text{g O}_2/\text{cm}^2/\text{h}^1 \right] = \frac{\Delta O_2 [\mu\text{g L}^{-1}] \times (V_{\text{beaker}} - V_{\text{coral}} [L])}{T \times \text{SA} \times \text{cm}^2}
\]

Corals were modeled as cylinders as this approximates their shape well; diameter and height were measured using a caliper. Coral volume \((V)\) and surface area \((A)\) were calculated following: Surface area \(A = \pi r^2\), Volume \(V = \pi hr^2\).

Additionally, we assessed the photochemical efficiency of \textit{F. granulosa} via PAM fluorometry (DIVING-PAM). The effective quantum yield (Genty \textit{et al.}, 1989):

\[
\Phi_{\text{PSII}} = \frac{F_{m} - F}{F_{m}} = \frac{\Delta F}{F_{m}}
\]

reflects the efficiency of photosystem II (PSII) under ambient light adapted conditions (Ralph & Gademann 2005). Ambient light conditions were logged with the PAM’s fiber quantum sensor. The light intensity for the incubation experiment (about 120 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) represents roughly the measured light intensity at the collection site (15 m; 100-150 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)).

4.3.5. \textbf{16S rRNA gene sequencing}

Flash frozen corals were stored at -80°C until DNA extraction. Each specimen was carefully unwrapped on ice, transferred into a sterile zip-lock bag, and doused with 5 mL
Qiagen RLT buffer (Qiagen AllPrep kit, Hilden, GER). While thawing, buffer and coral tissue were carefully blasted off using tap air pressure and pipette barrier tips. The buffer-tissue mixture was transferred into 15 mL Falcon tubes and vortexed. A 500 µL aliquot was used for DNA extraction following the manufacturer’s protocol (Qiagen AllPrep kit). DNA concentrations were quantified on a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Water samples were transported to the lab on ice in the dark. We filtered 1 L of each water sample over 0.22 µm Durapore PVDF filters (Millipore, Billerica, MA, USA). Filters were frozen at -80°C. Half of each filter was cut into small stripes with sterile razorblades and transferred into 2 mL Eppendorf vials. After adding 400 µl Qiagen RLT buffer, the samples were incubated on a rotating wheel for 20 min. Subsequent extraction steps were conducted following the manufacturer’s protocol (Qiagen AllPrep kit).

For PCR amplification we used about 60 ng DNA for coral samples and about 5 ng DNA for water samples. We used the primers 784F [5'- TCGTCGCCCAGCAGCATGTTATAAGAGACAGAGGATTAGATAACCCTGGTA-3’] an 1061R [5’- GTCTCGTTGGCTCGGAGATGTGTATAAGAGACAGCRCCAGAGCTGACGAC-3’] that target variable regions 5 and 6 of the 16S rRNA gene (Andersson et al., 2008) and have been shown to amplify well with coral DNA (Bayer et al., 2013). The primers contain Illumina adapter overhangs (underlined above; Illumina, San Diego, CA, USA). All PCRs were performed in triplicates using Qiagen Multiplex PCR kit with 0.2 µM of each primer adjusted to a total volume of 20 µl with RNase free water. The amplification cycling temperatures were set to one cycle at 95°C for 15 min, 25 cycles each at 95°C for 30 s, 55°C for 90 s, and 72°C for 30 s; a final extension step at 72°C for 10 min. 10 µL of each sample were used for visual quality check via 1% agarose gel electrophoresis. The triplicate PCRs of each sample were then pooled and cleaned with Agencourt AMPure XP
magnetic bead system (Beckman Coulter, Brea, CA, USA). The clean PCR product then underwent an indexing PCR to add Nextera XT indexing and sequencing adapters (Illumina) according to the manufacturer’s protocol. Indexed PCR products were cleaned up using the Agencourt AMPure XP protocol, quantified on the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and QuBit (Quant-IT dsDNA Broad Range Assay Kit; Invitrogen, Carlsbad, CA, USA), and pooled in equimolar ratios. The final pooled library was purified on a 2% agarose gel to remove any excess primer dimers. The library was sequenced at 8 pM with 10% phiX on the Illumina MiSeq, 2 x 300 bp paired end version 3 chemistry according to the manufacturer’s specifications. Sequences determined in this study have been deposited in the NCBI Sequence Read Archive under accession number PRJNA282461. (http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA282461)

4.3.6. **Bacterial community analysis**

For amplicon analysis, we used mothur (http://www.mothur.org/ version 1.16.1, Schloss et al., 2009). Paired end sequence reads were split according to barcodes, assembled to contigs, and quality trimmed. Duplicates, i.e. identical sequences, were merged using the ‘unique.seqs’ command in order to save computation time, and the command ‘count.seqs’ were used to keep a count of the number of sequences over samples represented by the remaining representative sequence. Rare sequence reads were removed (n<10 over all samples), remaining sequences were aligned against SILVA (release 119, Pruesse et al., 2007), and a pre-clustering step (2 bp difference) was performed (Huse et al., 2010). Chimeric sequences were removed using UCHIME as implemented in mothur (Edgar et al., 2011). Additionally, chloroplasts, mitochondria, archaea, eukaryotes, and unknown reads were removed. Sequences were classified against Greengenes (McDonald et al.,
2012) using a bootstrap of 60, and the sample compositions were compared on a family level. For further analyses, sequences were subsampled to 10,000 sequence reads and a 97% similarity cutoff level was chosen to obtain OTUs. Chao1 (Chao 1984), Simpson Evenness, Inverse Simpson Index, Principal Coordinate Analysis (PCoA), and Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) were performed as implemented in mothur. PCoA results were plotted in SigmaPlot 11. To identify the main contributing OTU for similarity within each of the groups, (i.e. freshly collected coral, short-term ambient salinity, short-term high salinity, long-term ambient salinity, and long-term high salinity), a Similarity Percentages (SIMPER) analysis was performed using PRIMER v6 software (Clarke & Gorley 2006). To identify OTUs that were significantly different between long-term hypersaline conditions and all other treatments we used the statistical package indicspecies (Cáceres & Legendre 2009) in R (R Core Team 2014). We employed the BEST (BIO-ENV) routine in PRIMER in order to identify the ‘best’ match between OTU distributions and associated environmental variables (i.e., salinity, temperature, dissolved oxygen, light levels, effective quantum yield, and depth). OTU abundances and environmental data were square root transformed and environmental data were normalized. Bray-Curtis similarity was used to calculate an OTU resemblance matrix, Euclidean distance was used for the environmental parameter resemblance matrix. The Weighted-Spearman rank correlation method was used to identify the parameter (or combination of parameters) providing the highest $\rho$.

To assess putative functional profiles based on the 16S community composition, we used METAGENassist for automated taxonomic-to-phenotypic mapping (Arndt et al., 2012). Input files were created in mothur using the ‘make.shared’ and ‘classify.otu’ commands based on all coral samples. During data processing in METAGENassist, all 2152 distinct OTUs were assigned, mapped, and condensed into 400 functional taxa and filtered based
on interquantile range (Hackstadt & Hess 2009). After filtering, 360 functional taxa remained and were normalized over samples by sum and over taxa by range scaling. We then analyzed the data for ‘metabolism-by-phenotype’, and used Euclidean distance measure and average clustering algorithm to visualize the results in a heatmap. To confirm patterns obtained by METAGENassist, we investigated specific genes associated with identified processes via PICRUSt (Langille et al., 2013). Input files for PICRUSt were created in mothur using the ‘make.biom’ command including all coral samples. Clusters of orthologous groups were created by predicting the metagenomes in PICRUSt.

4.4. Results

4.4.1. Coral and Symbiodinium physiology after short-term hypersalinity treatment

To assess short-term effects of strongly increased salinity on coral holobiont function we determined physiological parameters from the coral host and Symbiodinium at the end of 4 h incubations in ambient (39) and hypersaline treatments (55) (Table 2). We could not visually detect any signs of bleaching (Table 2), but we observed increased mucus production including small bubbles in the high salinity treatments (Supplementary Figure S1). Corals displayed an about eight-fold decreased calcification rate (G) under hypersaline compared to ambient conditions (0.031±0.073 compared to 0.243±0103 CaCO₃ µmol cm⁻² h⁻¹, \( P_{\text{test}} < 0.05 \)). Oxygen net production (i.e. oxygen-producing photosynthesis mainly by Symbiodinium, respiration mainly by coral host and Symbiodinium, bacterial contribution to both) was higher under ambient (start: 8.03±0.05 mgL⁻¹; stop: 10.47±1.15 mgL⁻¹) than under hypersaline (start: 7.14±0.11 mgL⁻¹; stop: 8.44±0.45 mgL⁻¹) conditions. Net photosynthesis-based oxygen production (\( P_n \)) was
significantly higher in ambient condition (13.49±4.87 compared to 8.28±3.98 µg O2/cm2/h, \(P_{\text{t-test}}<0.05\)). As expected, the effective quantum yield (\(\phi_{\text{PSII}}\)) of PSII in \textit{Symbiodinium} showed no distinct differences between corals from ambient and high salinity conditions at the experiment start (0.709±0.019 and 0.710±0.019). In contrast, \(\phi_{\text{PSII}}\) was significantly decreased for the hypersalinity treatment after the 4 h incubation period (0.681±0.018 compared to 0.718±0.018 under ambient conditions, \(P_{\text{t-test}}<0.05\)).

### Table 2. Physiological response of coral host and algal symbiont to short- and long-term hypersalinity exposure (* = \(P_{\text{t-test}}<0.05\); NA = not available, values are shown as means±SD).

<table>
<thead>
<tr>
<th></th>
<th>short-term (4 h)</th>
<th>long-term (29 d)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>Calcification rate</td>
<td>0.243±0.103</td>
<td>0.031±0.073*</td>
</tr>
<tr>
<td>[CaCO(_3) µmol cm(^{-2})h(^{-1})]</td>
<td></td>
<td></td>
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<tr>
<td>Photosynthetic efficiency</td>
<td>(\phi_{\text{PSII}})</td>
<td>0.718±0.018</td>
</tr>
<tr>
<td>Coral tissue discoloration</td>
<td>Not visually apparent</td>
<td>Not visually apparent</td>
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<td>(bleaching)</td>
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</table>

#### 4.4.2. Coral and \textit{Symbiodinium} physiology after long-term hypersalinity treatment

\textit{F. granulosa} specimens showed no signs of impaired photosynthetic performance during and after a 29 day \textit{in situ} experiment (van der Merwe \textit{et al.}, 2014b). We found a rapid dilution of the discharged brine: Only salinity levels at station 1 (i.e. at the SWRO discharge screen) were increased (average 49.4±2.0) and significantly (\(P_{\text{ANOVA}}<0.05\)) different from all other stations (2-6), where salinity was on average 41.3±0.7 and not significantly different between stations (\(P_{\text{ANOVA}}>0.05\)). Therefore, corals from station 1 were assigned to the hypersalinity long-term treatment (n = 3) and corals from all other
stations were pooled into the long-term ambient group (n = 15). Water temperature decreased slightly with increasing distance from the discharge, but differences did not exceed 0.5°C across average temperatures (stations 1-6, AVG: 26.3°C, 26.1°C, 26.0°C, 26.0°C, 25.8°C, 25.8°C). Dissolved oxygen varied between 5.75 and 6.37 mgL⁻¹ and was not significantly different ($P_{\text{ANOVA}}$>0.05) between stations. *Symbiodinium* effective quantum yields ($\phi_{\text{PSII}}$) were stable for all corals regardless of high or ambient salinity conditions. Average $\phi_{\text{PSII}}$s were within the natural range measured at the collection site (0.682-0.709) with 0.705±0.009 (station 1), 0.694±0.008 (station 2), 0.687±0.007 (station 3), 0.687±0.019 (station 4), 0.693±0.017 (station 5), and 0.690±0.02 (station 6) (see section 3.4.). Differences across the stations - and between daily measurements - coincided with light levels as assessed by PAR values (van der Merwe *et al.*, 2014b), as shown by a linear regression analysis ($R^2 = 0.80$, $P<0.001$). $\phi_{\text{PSII}}$ measurements from station 1, reflecting ambient levels from the collection site, indicate that high salinity did not impair the photosynthesitical efficiency of *Symbiodinium in hospite*.

### 4.4.3. Seawater and coral bacterial communities

We produced 41 16S rRNA gene libraries with a total of 4 784 475 reads distributed over 9 water samples, 4 freshly collected *F. granulosa* colonies, 10 short-term incubation corals (5 high and 5 ambient salinity), and 18 long-term treatment corals (3 high salinity at station 1; 15 ambient, i.e. 3 from each of stations 2-6). After quality trimming, chimera detection, and removal of undesired (e.g. chloroplasts) and rare sequences (n<10 over all samples), 2 612 132 reads with an average length of 292 bp were retained for further analyses. In order to assess sample and condition specific differences in bacterial composition we classified all sequences to the family level (Figure 7). Water samples were
dominated by *Pelagibacteriaceae* (~60-70%) and markedly different from all coral samples, but similar to each other. Coral samples were dominated by bacteria from the family *Rhodobacteraceae* (~20-80%), except for long-term hypersalinity coral samples, which appeared more even and were not dominated by a distinct bacterial family (Figure 7).
Figure 7. Bacterial taxonomy stack plot on the phylogenetic level of family (Greengenes database, bootstrap≥60). Each color represents one of the 16 most abundant families in all samples. All other taxa are grouped under category ‘others’. ST = short-term incubation; LT = long-term transect; A = ambient; H = hypersaline; numbers in the LT sample names denote transect station; WS = water sample; asterisks denote hypersaline samples.
4.4.4. Coral microbiome restructuring after long-term salinity exposure

To reveal differences in bacterial community composition between treatments and over time, we subsampled to 10,000 reads and clustered sequence data to operational taxonomic units (OTUs, similarity cutoff ≤ 0.03). Good's estimator (Good 1953) ranged between 0.98 and 1.0 coverage for all samples indicating that the majority of bacterial diversity was represented. Chao1 estimates of species richness, Simpson evenness, and Inverse Simpson Index (diversity measure) were all highest for long-term hypersalinity samples compared to all other samples - with an approximate average ten-fold increase for diversity (Inverse Simpson Index) and a three-fold increase for Chao1 and Simpson Evenness (Table 3). We compared microbial community abundance profiles over coral colonies, treatments, and time points in a Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity (Figure 8). Water samples were clustering closely together and differed significantly from all coral samples \((P_{AMOVA} \leq 0.005)\), but not between each other \((P_{AMOVA} = 1)\) confirming that bacterial communities from hypersaline water samples did not exhibit differences in comparison to ambient water samples (Supplementary Figure S2). Importantly, we observed a distinct grouping among coral samples. Long-term hypersaline coral samples clustered significantly away from all other coral samples (i.e. freshly collected, short-term incubations, and long-term ambient) \((P_{AMOVA} \leq 0.001)\), and all other coral samples were not significantly different from each other \((P_{AMOVA} \geq 0.05)\). In order to uncover the main environmental driver underlying the coral microbiome changes, we employed a biological-environmental matching routine that compares OTU distribution to environmental parameters. This analysis revealed a significant correlation between environmental data (i.e., salinity, temperature, dissolved oxygen, light levels, effective quantum yield, and depth) and the coral microbiomes. From all considered environmental parameters and combinations thereof, salinity as a single factor best...
explained variations in OTU abundance and distribution ($\rho = 0.639$, $P_{\text{BEST}} \leq 0.01$) (Supplementary Table S1).
Table 3. Summary statistics of 16S rRNA gene amplicon sequencing detailing reef water and microbial communities associated with *Fungia granulosa* under ambient and hyper-saline conditions (* = subsampled to n = 10000; ST = short-term incubation; LT = long-term incubation; A = ambient; H = hypersaline; Numbers in the LT sample names denote transect station; WS = water sample). Total number of OTUs = 2,235.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>Treatment</th>
<th>No. of reads</th>
<th>Coverage*</th>
<th>No. of OTUs (total 2235)*</th>
<th>Chao1*</th>
<th>Simpson Evenness*</th>
<th>Simpson Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>reef water</td>
<td>reef coral_R1</td>
<td>-</td>
<td>105,511</td>
<td>1.00</td>
<td>129</td>
<td>161</td>
<td>0.021</td>
<td>2.646</td>
</tr>
<tr>
<td></td>
<td>reef coral_R2</td>
<td>-</td>
<td>29,560</td>
<td>1.00</td>
<td>98</td>
<td>119</td>
<td>0.025</td>
<td>2.408</td>
</tr>
<tr>
<td></td>
<td>reef coral_R3</td>
<td>-</td>
<td>20,358</td>
<td>1.00</td>
<td>61</td>
<td>80</td>
<td>0.027</td>
<td>1.661</td>
</tr>
<tr>
<td></td>
<td>reef coral_R4</td>
<td>-</td>
<td>158,984</td>
<td>0.99</td>
<td>168</td>
<td>260</td>
<td>0.014</td>
<td>2.343</td>
</tr>
<tr>
<td>short-term ambient salinity</td>
<td>ST_A_R1</td>
<td>4h at 39</td>
<td>92,228</td>
<td>0.99</td>
<td>206</td>
<td>237</td>
<td>0.019</td>
<td>3.984</td>
</tr>
<tr>
<td></td>
<td>ST_A_R2</td>
<td>4h at 39</td>
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<td>98</td>
<td>129</td>
<td>0.040</td>
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</tr>
<tr>
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<td>4h at 39</td>
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<td>0.023</td>
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<tr>
<td></td>
<td>ST_A_R5</td>
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<td>1.00</td>
<td>84</td>
<td>111</td>
<td>0.033</td>
<td>2.814</td>
</tr>
<tr>
<td>short-term hypersaline</td>
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<td>1.00</td>
<td>113</td>
<td>118</td>
<td>0.041</td>
<td>4.685</td>
</tr>
<tr>
<td></td>
<td>ST_H_R2</td>
<td>4h at 55</td>
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<td>0.99</td>
<td>174</td>
<td>223</td>
<td>0.022</td>
<td>3.809</td>
</tr>
<tr>
<td></td>
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<td>4h at 55</td>
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<td>69</td>
<td>96</td>
<td>0.022</td>
<td>1.534</td>
</tr>
<tr>
<td></td>
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<td>0.077</td>
<td>10.670</td>
</tr>
<tr>
<td></td>
<td>ST_H_R5</td>
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<td>125</td>
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<td>4.050</td>
</tr>
<tr>
<td>long-term ambient salinity</td>
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<td>29d at ~41</td>
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<td>348</td>
<td>525</td>
<td>0.009</td>
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<tr>
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<td>LT_A_2_R3</td>
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<td>300</td>
<td>0.009</td>
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<td>376</td>
<td>0.035</td>
<td>10.420</td>
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<td>15,655</td>
<td>0.99</td>
<td>282</td>
<td>334</td>
<td>0.015</td>
<td>4.188</td>
</tr>
<tr>
<td></td>
<td>LT_A_3_R3</td>
<td>29d at ~41</td>
<td>59,872</td>
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<td>141</td>
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<td>0.017</td>
<td>2.391</td>
</tr>
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<td>LT_A_4_R1</td>
<td>29d at ~41</td>
<td>63,348</td>
<td>0.99</td>
<td>272</td>
<td>314</td>
<td>0.033</td>
<td>9.006</td>
</tr>
<tr>
<td></td>
<td>LT_A_4_R2</td>
<td>29d at ~41</td>
<td>123,875</td>
<td>0.99</td>
<td>310</td>
<td>495</td>
<td>0.011</td>
<td>3.317</td>
</tr>
<tr>
<td></td>
<td>LT_A_4_R3</td>
<td>29d at ~41</td>
<td>72,038</td>
<td>0.99</td>
<td>276</td>
<td>399</td>
<td>0.017</td>
<td>4.752</td>
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<tr>
<td></td>
<td>LT_A_5_R1</td>
<td>29d at ~41</td>
<td>68,602</td>
<td>0.99</td>
<td>264</td>
<td>371</td>
<td>0.011</td>
<td>2.823</td>
</tr>
<tr>
<td></td>
<td>LT_A_5_R2</td>
<td>29d at ~41</td>
<td>106,800</td>
<td>0.98</td>
<td>446</td>
<td>631</td>
<td>0.009</td>
<td>4.147</td>
</tr>
<tr>
<td></td>
<td>LT_A_5_R3</td>
<td>29d at ~41</td>
<td>55,622</td>
<td>0.99</td>
<td>228</td>
<td>279</td>
<td>0.008</td>
<td>1.903</td>
</tr>
<tr>
<td></td>
<td>LT_A_6_R1</td>
<td>29d at ~41</td>
<td>121,617</td>
<td>0.99</td>
<td>228</td>
<td>377</td>
<td>0.019</td>
<td>4.338</td>
</tr>
<tr>
<td></td>
<td>LT_A_6_R2</td>
<td>29d at ~41</td>
<td>47,763</td>
<td>0.99</td>
<td>265</td>
<td>327</td>
<td>0.013</td>
<td>3.565</td>
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<tr>
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<td>LT_A_6_R3</td>
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<td>64,844</td>
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<td>13.754</td>
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<tr>
<td>long-term hypersaline</td>
<td>LT_H_1_R1</td>
<td>29d at ~49</td>
<td>32,455</td>
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<td>608</td>
<td>685</td>
<td>0.091</td>
<td>55.242</td>
</tr>
<tr>
<td></td>
<td>LT_H_1_R2</td>
<td>29d at ~49</td>
<td>63,823</td>
<td>0.98</td>
<td>736</td>
<td>835</td>
<td>0.076</td>
<td>56.261</td>
</tr>
<tr>
<td></td>
<td>LT_H_1_R3</td>
<td>29d at ~49</td>
<td>56,052</td>
<td>0.98</td>
<td>864</td>
<td>968</td>
<td>0.042</td>
<td>36.288</td>
</tr>
</tbody>
</table>
Figure 8. Clustering of coral samples based on Bray-Curtis dissimilarity of microbial community abundances in a Principal Coordinate Analysis (PCoA) ($R^2 = 0.91$). Water samples = reef water, reef = freshly collected *F. granulosa*, short-term ambient = 4 h at 39, short-term hyper = 4 h at 55, long-term ambient = 29 d at 41, long-term hyper = 29 d at 49.

To assess overall similarity and to identify the main contributing OTU within each of the groups (i.e. freshly collected coral, short-term ambient salinity, short-term hypersalinity, long-term ambient salinity, and long-term hypersalinity), we looked for the presence of 'core' microbiome OTUs with SIMPER (Similarity Percentages) analysis. All groups showed a consistent 'within-group' similarity of about 50% (Bray-Curtis) (Table 4). Looking at the main contributing OTU within long-term hypersaline samples, we identified OTU0010 (*Pseudomonas veronii*) (Table 4). The average abundance of this OTU was increased by about three-fold in corals from the long-term hypersaline treatment compared to all other coral samples (836.7 vs. 270.7 average read counts) (Supplementary File S3). Strikingly, corals from all other groups revealed the same single, unclassified OTU in the family *Rhodobacteraceae* (OTU0001) to be the numerically
abundant bacterial taxon (Table 4). The average abundance of OTU0001 for these groups was 5105.6 reads (relative abundance 51.1%) compared to an average abundance of only 133 reads (relative abundance 1.33%) of this OTU in long-term hypersaline coral samples (Table 4; Supplementary File S3).

Table 4. Summary of SIMPER analyses showing the main contributing OTU in each treatment group. Displayed are Bray-Curtis similarity measures between group members based on OTU abundances within a sampling group, the main contributing OTU, the average abundance of this OTU, and the average contribution of this OTU to the overall group similarity.

<table>
<thead>
<tr>
<th>Treatment group (Bray-Curtis similarity)</th>
<th>main contributing OTU (bootstrap value)</th>
<th>AVG read abundance</th>
<th>AVG contribution [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly collected coral (51.19%)</td>
<td>OTU0001 unclassified sp.(100), family Rhodobacteraceae(100)</td>
<td>6530</td>
<td>36.53</td>
</tr>
<tr>
<td>Short-term ambient (49.98%)</td>
<td>OTU0001 unclassified sp.(100), family Rhodobacteraceae(100)</td>
<td>5627.2</td>
<td>30.65</td>
</tr>
<tr>
<td>Short-term hypersalinity (47.95%)</td>
<td>OTU0001 unclassified sp.(100), family Rhodobacteraceae(100)</td>
<td>4594.4</td>
<td>23.42</td>
</tr>
<tr>
<td>Long-term ambient (44.03%)</td>
<td>OTU0001 unclassified sp.(100), family Rhodobacteraceae(100)</td>
<td>4722.3</td>
<td>19.29</td>
</tr>
<tr>
<td>Long-term hypersalinity (56.20%)</td>
<td>OTU0010 Pseudomonas veronii(93), family Pseudomonadaceae(100)</td>
<td>836.7</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Based on the similarity of diversity estimates, proximity in PCoA clustering, and an identical main contributing OTU for the microbial communities of freshly collected coral, short-term ambient salinity, short-term high salinity, and long-term ambient salinity treatments, we jointly compared corals from these treatments to corals from the long-term high salinity treatment. Differentially abundant OTUs were determined with indicspecies and revealed that 523 OTUs were significantly (P≤0.01) different between corals from the hypersaline long-term treatment in comparison to all other coral samples. Interestingly, only 3 OTUs (of a total of 4 OTUs) were highly significantly (P≤0.001)
enriched in all coral groups, but absent in corals from the hypersaline long-term treatment. These were OTU0003, OTU0005 (both unclassified spp., order Cytophagales), and OTU0009 (unclassified sp., family Rhodobacteraceae) (Supplementary Table S2). In contrast, indicspecies identified 519 significantly enriched OTUs in corals from the hypersaline long-term treatment, which together made up a relative abundance of 55.6%. Of those, 5 OTUs had an average abundance of at least 100 reads and 104 OTUs had an average abundance of at least 10 reads and were highly significantly enriched in corals from the hypersaline long-term treatment (P≤0.001; Supplementary Table S2). The highly significant enrichment of hundreds of OTUs under hypersaline conditions over a course of 29 days in connection with the parallel decrease of the otherwise numerically abundant OTU (i.e. OTU0001) demonstrates a major restructuring of the coral microbiome. In contrast to previous findings (Bouvier & del Giorgio 2002), bacterial communities in the seawater did not change upon high salinity exposure, presumably because of a rapid brine dilution (van der Merwe et al., 2014b) and thus short residence times of associated bacteria therein.

4.4.5. Taxonomy-based functional profiling of bacterial communities

We used METAGENassist to predict putative changes in bacterial community function based on differences in bacterial community composition. In line with our taxonomy-based analysis, the long-term hypersaline samples formed a distinct group from all other coral samples (Figure 9). In these three samples we found a pronounced upregulation of polyhydroxybutyrate storage, sulfur oxidizer, syntrophic bacteria, and xylan, alkane, and biomass degraders (Figure 9). At the same time dehalogenation, ammonium oxidizer, and nitrite and sulfate reducer were down-regulated. Next, we used PICRUSt to analyze specific genes underlying identified processes (i.e., PHB metabolism, sulfur cycling, and
nitrogen cycling) affected in the long-term hypersaline samples. Further supporting the results from METAGENassist, we found decreased abundance of the enzyme polyhydroxybutyrate depolymerase associated with PHB metabolism, decreased anaerobic DMSO reductase and sulfite reductase associated with sulfur cycling, and decreased nitrite reductase associated with the process of nitrite reduction (Supplementary Figure S3). Besides these processes, some samples were enriched for other functions, e.g. ‘iron oxidizer’, ‘sugars fermentor’, ‘chitin degradation’, ‘selenate reducer’ (among others) in METAGENassist. However, samples enriched for these functions did not reveal any apparent patterns in regard to treatment (i.e. control, ambient, or high salinity) or exposure time (i.e., 4 h or 29 days).
Figure 9. Taxonomy-based functional profiling of bacterial communities. Heatmap created in METAGENassist displaying changes in putative functional profiles based on the 16S community composition. Changes are displayed on a relative scale with enrichment in red and depletion in blue. Data were analyzed for metabolism by phenotype with an Euclidean distance measure and average clustering algorithm. ST = short-term incubation; LT = long-term treatment; A = ambient; H = hypersaline; Numbers in the LT sample names denote transect station.
4.5. Discussion

In this study we assessed the effect of short- and long-term hypersalinity exposure on the Red Sea coral *Fungia granulosa*. Our results indicate distinct short- and long-term reactions of the coral holobiont. The short-term experiment, aimed to measure the initial response, was characterized by an absence of changes in the bacterial community structure, but a significant reduced calcification and photosynthesis under strongly increased salinity levels. In contrast, the long-term transect experiment indicated a putative acclimatization response, since corals exposed to high salinity for 29 days did not exhibit measureable (photo) physiological effects or signs of bleaching (van der Merwe *et al.*, 2014b), but rather, displayed a significant shift in the associated bacterial community.

4.5.1. Short-term and long-term coral physiology

*Fungia granulosa* is a single-polyp scleractinian coral that has been demonstrated to possess a slow growth rate (Chadwick-Furman *et al.*, 2000). Accordingly, our values for the calcification rate (G) were lower than those reported for other corals, e.g. for *Stylophora subseriata* (1.05-1.73 µmol CaCO$_3$ cm$^{-2}$h$^{-1}$) (Sawall *et al.*, 2011). It is of note that calcification effectively stopped at high salinities in the short-term treatment. Unfortunately, calcification rates are not available for the long-term treatment. However, it would be interesting to see whether calcification rates are not influenced in long-term hypersaline exposure – as observed for photosynthetic efficiency.

In line with previous studies, our short-term incubation showed an overall oxygen increase, which was significantly lower for high salinity conditions (Gattuso *et al.*, 1999; Lirman & Manzello 2009; Manzello & Lirman 2003). Further, a reduction of photosynthetic rates has been documented for hyper- and hyposaline scenarios before
(Alutoin et al., 2001; Chartrand et al., 2009; Ferrier-Pages et al., 1999; Moberg et al., 1997; Muthiga & Szmant 1987). However, long-term $\phi$PSII values from our coral samples were in the same range as photosynthetic yields originating from corals in their natural environment. We could not observe any differences between hypersaline and ambient conditions in regard to the coral’s photophysiology or bleaching status (van der Merwe et al., 2014b).

In accordance with data collected from our short- and long-term experiments, an initial sharp decline in photosynthetic performance with subsequent recovery has been suggested as an acclimatization pattern (Lirman & Manzello 2009; Manzello & Lirman 2003). Mayfield and Gates (2007) interpreted these patterns as an indication for osmoregulatory processes, also considering corals to generally tolerate slow salinity changes better than more rapid ones (Muthiga & Szmant 1987). Our physiological data are supporting corals as being able to adjust to salinity. We report distinct physiological effects on coral host (calcification) and Symbiodinium (decreased photosynthesis) in our 4 h incubation. In the long-term in situ experiment we could not find any influence on the (photo) physiology of F. granulosa indicating acclimatization of the coral holobiont to prevailing salinity levels.

4.5.2. Coral microbiome restructuring

To our knowledge, this is the first study that assesses coral bacterial microbiome structure under short- and long-term exposure of corals to salinity changes. Healthy corals maintain mostly specific, stable, and uneven microbial assemblages indicating selected microbimes (Bayer et al., 2013; Bourne et al., 2008; Bourne & Webster 2013; Jessen et al., 2013; Kelly et al., 2014; Meron et al., 2011a; Meron et al., 2012). The microbial
communities are diverse and contribute to pathogen inhibition due to production of antimicrobial substances as well as competition for space and nutrients (Klaus et al., 2007; Rosenberg et al., 2007; Thurber et al., 2009). In our experiments we found no distinct bacterial community changes after 4 h salinity exposure, which contrasts the measured physiological reactions of coral host and algal symbiont. At the same time, Apprill et al., (2009) measured doubling times of 10+ hours for coral associated bacteria, which may have affected our ability to determine a microbiome response in the short-term experiment. Conversely, we found no physiological reaction, but pronounced microbial community changes after a 29 d hypersaline treatment. All corals, except those from the long-term hypersaline treatment, revealed highly uneven bacterial microbiomes that were numerically dominated by a single, distinct OTU (i.e. OTU0001) that could only be identified to the level family, namely *Rhodobacteraceae*. Bacteria from this family were repeatedly observed in healthy corals (Bayer et al., 2013; Ceh et al., 2012; Kellogg et al., 2014; Li et al., 2014; Morrow et al., 2012; Sunagawa et al., 2009), even though they have also been found to be associated with stressed corals and stressed sea urchins (Buchan et al., 2005; Godwin et al., 2012; Meron et al., 2011a; Meron et al., 2011b; Meron et al., 2012; Pantos et al., 2015; Sunagawa et al., 2009). Additionally, bacteria in the family *Rhodobacteraceae* have been found to be enriched in corals isolated from deeper habitats (27 m) compared to their shallow counterparts (6 m) (Pantos et al., 2015), which may explain their dominance in healthy *F. granulosa* collected in this study from a depth of 15-18 m. Taken together, the presence of *Rhodobacteraceae* in a range of hosts denotes environmental flexibility. For this reason, it is challenging to assign a specific role. However, the high abundance of a distinct OTU of this bacterial family in *F. granulosa* specimens from all treatments but the high salinity long-term treatment indicates that this taxon likely provides an important function to the coral holobiont.
In contrast, *Pseudomonas veronii*, the ‘core’ microbiome member and the most abundant taxon in corals from the hypersaline long-term treatment, was present at a much lower abundance in corals from all other treatments (i.e. freshly collected coral, short-term ambient salinity, short-term high salinity, and long-term ambient salinity). Since *P. veronii* was present in all corals, albeit at lower abundance, we argue that its increase under high salinity might signify a change of selection regime for this taxon under the altered environmental conditions, and not an opportunistic association. The uniformity of all water samples, i.e. no significant differences in water samples over different treatments or time points, further supports a selective process for the changes in the coral microbiomes. The specific function of *P. veronii* remains to be determined. However, it seems to be a versatile taxon that has been isolated from distinct environments, e.g. natural freshwater springs, soil samples, and wastewater filters where it has been shown to degrade a variety of simple aromatic organic compounds making it a beneficial bacterium for bioremediation of contaminated environments (Elomari *et al.*, 1996; Nam *et al.*, 2003; Onaca *et al.*, 2007). More generally, bacteria in the genus *Pseudomonas* have repeatedly been shown to be abundant in hypersaline environments and display broad metabolic capacity (Brusa *et al.*, 2001; Fendrich 1988; Isnansetyo & Kamei 2009; Sass *et al.*, 2001).

Among other bacterial taxa that increased in abundance in the long-term hypersalinity treatment, we identified the coral pathogen *Vibrio shilonii* (OTU0264) and also some unclassified *Alteromonadaceae* taxa (Supplementary Table S2). These taxa are presumedly associated with coral stress and disease, but are known to reside in healthy corals as well (Rosenberg & Falkovitz 2004; Sunagawa *et al.*, 2009). Taken together, bacterial microbiome restructuring under high salinity levels as signified by loss of the numerically dominant bacterial taxon (i.e. OTU0001), the increase in *P. veronii* (i.e.
OTU0010), as well as an overall increase in richness, evenness, and diversity possibly indicates stress (Bourne et al., 2008; Garren et al., 2009; Meron et al., 2011a; Meron et al., 2011b; Sunagawa et al., 2009; Zhang et al., 2015). At the same time, major microbiome restructuring induced by environmental stress (i.e. high salinity) in the absence of a measurable physiological reaction of the coral holobiont may give support to the probiotic hypothesis (Reshef et al., 2006), i.e. a change of the microbiome to facilitate coral holobiont acclimatization.

4.5.3. Functional changes of bacterial communities indicate metabolic adjustment

Mapping of differences in bacterial community composition to putative functional differences revealed a prominent increase in polyhydroxybutyrate (PHB) storage as well as changes in nitrogen and sulfur cycling in long-term hypersaline samples in comparison to all other coral samples. PHB can be synthesized by microorganisms as a carbon reservoir in cells (Roberts 2005) and may be produced in response to variable stressors, such as nutrient limitation, e.g. under nitrogen limiting conditions (Ayub et al., 2004; Soto et al., 2012). Interestingly, PHB has also been identified as an osmolyte in microorganisms (Arora et al., 2006; Doronina et al., 2000; Martin et al., 2002; Soto et al., 2012). Additionally, PHB production in rhizobia bacteria with a potential benefit for plant cultivation in saline soil has been suggested (Ali et al., 2014; Arora et al., 2006). It is striking that Pseudomonas strains closely related to P. veronii are shown to produce PHB (Ayub et al., 2004; Soto et al., 2012; Yan et al., 2008), but even more so, the genome of P. veronii harbors the enzyme 3-hydroxyisobutyryl dehydrogenase (Ramírez-Bahena et al., 2015), which is part of the PHB metabolism (Hügler & Sievert 2011). This provides a
putative functional link to the numerical dominance of *P. veronii* in the long-term hypersaline samples and potentially indicates functional adaptation of the coral holobiont by alteration of its microbiome. Such functional changes were shown in the aphid *Acyrthosiphon pisum* where replacing the native gut bacteria *Buchnera* line LSR1 with line 5AY from a more thermotolerant aphid matriline conferred a dramatic increase in thermal tolerance (Moran & Yun 2015).

Changes in sulfur cycling as suggested by an up-regulation of ‘Sulfur oxidizer’ and a down-regulation of ‘Sulfate reducer’ presumably indicate the enrichment of oxidized products in the sulfur metabolism. The coral holobiont is a major contributor to the production of dimethylsulphide (DMS), a central compound of the global sulphur cycle (Raina *et al.*, 2013), which can become oxidized to dimethylsulphoxide (DMSO) (Sunda *et al.*, 2002). DMSO has a stronger reactivity towards reactive oxygen species (ROS) than DMS, is more hydrophilic (allowing higher cellular concentrations), and can be further oxidized to the water-soluble antioxidant methane sulphinic acid (Sunda *et al.*, 2002). Hence, an increased production (accompanied by a decreased reduction) of DMSO acting as an ROS scavenger may enable the coral to cope with increased oxidative stress in *Symbiodinium*. In agreement with these patterns, increased oxidative stress accompanied by antioxidant production as a response to high salinity has been shown for algae and other plants (Fadzilla *et al.*, 1997; Gossett *et al.*, 1996; Hernández *et al.*, 2000; Jahnke & White 2003). Another distinct pattern emerged from the metabolic profile of nitrogen-related functions. We found processes that increase nitrogen availability for the holobiont to be enhanced (i.e., ‘dinitrogen-fixing’ and ‘nitrogen fixation’), whereas processes that require the availability of fixed nitrogen are reduced (i.e., ‘ammonia oxidizer’ and ‘nitrite reducer’). This suggests an enhanced nutrient limitation of the coral holobiont (Rädecker
et al., 2015). Nutrient limitation may be a consequence of an increased metabolism with enhanced nutrient requirements.

4.5.4. **Long-term coral holobiont response may indicate acclimatization**

Changes of the coral microbiome under changed environmental conditions were previously described (e.g. Jessen et al., 2013; Kelly et al., 2014; Klaus et al., 2007; Meron et al., 2012; Pantos et al., 2015), and that these changes are relevant to holobiont function was demonstrated by Moran & Yun (Moran & Yun 2015). In line with these studies, we interpret the here-observed prevalent change of the coral microbiome in combination with a lack of an apparent stress response by the coral or symbiont in the long-term high salinity treatment as indication for an acclimatization response. This is supported by the putative functional changes we detected in the microbial community, i.e. upregulation of PHB as an osmolyte, alterations to the nitrogen cycle to compensate for nutrient deficiency, and synthesis of DMSO as a ROS scavenger. It is important to consider that the adjustments of the *Fungia granulosa* coral holobiont to a high salinity environment presumably require considerable energy and these energy requirements need to be taken into account when assessing the response of corals to changes in salinity. Taking the large biomass of the solitary coral *F. granulosa* into account, energy reserves may be sufficient for supposedly initial stress periods (as a response to the changing environmental conditions) and simultaneous acclimatization. By comparison, commonly employed setups using small coral fragments in short-term experiments may considerably underestimate coral resilience towards (salt) stress and might miss acclimatization due to insufficient energy reserves of the coral fragment to sustain and acclimate to the
stressor (Chartrand et al., 2009; Ferrier-Pages et al., 1999; Kerswell & Jones 2003; Lirman & Manzello 2009; Manzello & Lirman 2003; Seveso et al., 2013).

Taken together, we argue that changes in salinities lead to (small) changes in the holobionts internal environment, which in turn affect microbiome structure by selecting for a more advantageous bacterial community composition as posited by the coral probiotic hypothesis (Reshef et al., 2006). Future studies should target the temporal stability of restructured coral microbiomes accompanied by physiological measures under enduring ‘stress’ conditions to unequivocally confirm the importance of the microbiome to coral holobiont function.

4.6. Author contributions & Acknowledgements

TR, CRV, and RvdM designed and conceived the experiments. TR and MAO generated data. TR, CRV, MAO, and AR analyzed and interpreted data. CRV and RvdM contributed reagents/materials/analysis tools. TR and CRV wrote the manuscript.

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4.7. References


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*COMP BIOCHEM PHYS A* 147, 1-10.


### 4.8. Supplementary Material

**Supplementary Figure S1.** Increased mucus production and bubble formation of the coral *Fungia granulosa* after 4 h incubation at (a) 39 (b) 55.

**Supplementary Figure S2.** Clustering of water samples based on Bray-Curtis dissimilarity of microbial community abundances in a principal coordinate analysis (PCoA) ($R^2 = 0.89$).
Supplementary Figure S3. Relative absence/presence of microbial genes associated with (A, B) PHB metabolism and (C, D) sulphur cycling via PICRUSt.

Supplementary Table S1. Correlation between coral microbiomes and environmental parameters.

<table>
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<th>$\rho$</th>
</tr>
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</tr>
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<td>Salinity, Effective quantum yield, PAR</td>
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</tr>
<tr>
<td>3</td>
<td>Effective quantum yield, PAR, Depth</td>
<td>0.629</td>
</tr>
<tr>
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Supplementary File S3 (in section 7. APPENDIX). OTU abundance counts over samples with annotation and reference OTU sequence.

Supplementary File S4 (in section 7. APPENDIX). OTUs enriched in fresh, short-term ambient and hypersaline, and long-term ambient corals ($P \leq 0.001$, average abundance $\geq 10$); OTUs enriched in long-term hypersaline corals ($P \leq 0.001$, average abundance $\geq 10$; abundance count in each of the three replicates).
CHAPTER III

5. The role of floridoside in osmoadaptation of coral algal endosymbionts to hypersaline stress

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5.1. Abstract

The endosymbiosis between *Symbiodinium* dinoflagellates and stony corals provides the foundation of coral reef ecosystems. The survival of these ecosystems is under threat at a global scale and better knowledge is needed to conceive strategies for mitigating future reef loss. Environmental disturbance imposing temperature, salinity, and nutrient stress can lead to the loss of the *Symbiodinium* partner, so-called coral bleaching. Some of the most thermotolerant coral-*Symbiodinium* associations occur in the Persian/Arabian Gulf and the Red Sea, which represent also the most saline coral habitats. We studied whether *Symbiodinium* alter their metabolite content in response to high salinity environments. We found that *Symbiodinium* cells exposed to high salinity produced high levels of the osmolyte 2-O-glycerol-α-D-galactopyranoside (floridoside), both in vitro and in their host animals, thereby increasing their capacity and putatively the capacity of the holobiont to cope with the effects of osmotic stress in extreme environments. Given that floridoside has been previously shown to also act as an antioxidant, this osmolyte may serve a dual function. Firstly, as a compatible organic osmolyte accumulated by *Symbiodinium* in response to elevated salinities, and secondly to counter reactive oxygen species (ROS) produced as a consequence of salinity stress.

5.2. Introduction

Coral reefs are biodiversity hotspots of immense biological and economical value (Reaka-Kudla *et al.*, 1996). The relationship between *Symbiodinium* – an autotroph endosymbiotic dinoflagellate – and scleractinian corals forms the basis of coral reef ecosystems (Muscatine and Porter, 1977). The coral host provides inorganic nutrients and carbon dioxide to the dinoflagellate in exchange for energy in the form of photosynthetically produced carbohydrates (Falkowski *et al.*, 1984; Muscatine *et al.*, 1989). This symbiotic
relationship is highly sensitive to environmental disturbance. For instance, increases in temperature, salinity, nutrients, and/or high solar irradiance can impair photosynthetic efficiency and enhance the formation of harmful reactive oxygen species (ROS) that must be detoxified by the organism via antioxidants and ROS scavengers (Brown, 1997; Lesser, 2011; Wiedenmann et al., 2013; Rädercker et al., 2015). Otherwise, the environmental stress can ultimately lead to coral bleaching, the visual whitening of corals due to loss of their endosymbionts (Brown, 1997; Lesser, 2011). As a consequence, increasing exposure to environmental stress, in particular to rising seawater temperatures, is threatening the existence of coral reefs at a global scale (Ainsworth et al., 2016; Bruno and Valdivia, 2016).

In comparison to the detrimental effects of elevated seawater temperatures, much less is known about the effects of increased salinities on corals and their endosymbionts. Yet, reef corals from the Red Sea and the Persian/Arabian Gulf (PAG) are commonly found at salinities of up to 41 in the Red Sea and up to 50 in the PAG at comparably high summer temperatures exceeding 32°C and 35°C in parts of the Red Sea and Persian/Arabian Gulf (PAG), respectively (Ngugi et al., 2012; D’Angelo et al., 2015; Hume et al., 2016; Röthig et al., 2016). In fact, both salinity and temperature in these regions are the globally highest to support reef growth (Coles, 2003). Although the osmotic response of Symbiodinium at the molecular level is virtually unknown (Goiran et al., 1997; Suoscún-Bolívar et al., 2012; Suoscún-Bolívar et al., 2016), studies on free-living algae suggest that production and accumulation of compatible organic osmolytes (COOs), referred to as osmoadaptation (Reed, 1984), is the most widespread mechanism for adjusting intracellular osmotic pressure in response to elevated salinity; accumulation of inorganic ions and cell volume changes may occur as well. However, since the latter two processes are stressful and can disturb cellular function, they are not considered to represent viable long-term solutions for osmoadaptation (Ahmad and Hellebust, 1984; Sadka et al., 1989; Kirst, 1990; Chen and
Jiang, 2009). In contrast, the accumulation of COOs adjust the osmotic pressure and protects proteins from increased ion concentrations (Kirst, 1990).

To test whether *Symbiodinium* is capable of synthesizing COOs, we subjected *Symbiodinium* strains from different clades and origins to high salinity stress, both *in vitro* and in their animal hosts (hereafter referred to as *in hospite*), and screened for the presence of COOs using gas chromatography-mass spectrometry (GC-MS). We hypothesized that *Symbiodinium* would increase cellular concentrations of COOs in response to elevated salinities and that the response would be similar *in vitro* as *in hospite*. Among the utilized COOs, we identified the carbohydrates floridoside, inositol, and mannitol *in vitro* and *in hospite*. These compounds can act as both osmolytes and antioxidants, thereby having the potential capacity to convey osmoadaptation to increased salinities and the ability to counter ROS produced as a consequence of salinity stress (Shen et al., 1997; Hasegawa et al., 2000; Li et al., 2010; Kim et al., 2013; Bose et al., 2014; Hoef-Emden, 2014).

5.3. **Materials and Methods**

5.3.1. *Symbiodinium* cultures

*Symbiodinium microadriaticum* CCMP2467 (type A1, originally isolated from *Stylophora pistillata*, Red Sea) (Aranda et al., 2016), *Symbiodinium* sp. type A1 (originally isolated from *Astreopora* sp., Red Sea) (Gong, 2012), *Symbiodinium minutum* Mf1.05b (type B1, isolated from *Orbicella faveolata*, FL, USA) (Bayer et al., 2012; Shoguchi et al., 2013), and *Symbiodinium psygmophilum* Mf10.14b.02 (type B2, isolated from *O. faveolata*, FL, USA) (LaJeunesse et al., 2012; Parkinson et al., 2016) were cultured in f/2 media without silicium under a photonflux of 108 µmol m⁻² s⁻¹ at 26°C (Reimann et al., 1963). The f/2
media was prepared from sterile filtered Red Sea water (salinity of 38, and complemented with NaNO$_3$, NaH$_2$PO$_4$, vitamins, and trace metals (Guillard and Ryther, 1962). For each strain, we used replicate culture flasks and prepared three salt-adjusted f/2 media (salinities: 25, 38, and 55, following (van der Merwe et al., 2014)) either by adding appropriate amounts of NaCl or dilution of the media with ddH$_2$O water. Triplicates of 5 mL of _Symbiodinium_ cultures at exponential growth ($10^5$-$10^6$ cells mL$^{-1}$) were transferred to 35 mL salt-adjusted f/2 media for each salinity and incubated for 4 h under culturing conditions. _Symbiodinium_ cells were subsequently harvested by centrifugation (4500 x g, 10 min, 4°C). Cells were counted by fluorescence-activated cell sorting (FACS). To do this, 1 ml of each _Symbiodinium_ culture were collected and fixed with formalin. After washing, samples were resuspended in 1 ml of ddH$_2$O and labeled with SYBR Gold (Thermo Fisher Scientific, USA), of which 150 µl aliquots were supplied for FACS (50 µl counting volume). FACS measurements were conducted in triplicate on a cell analyzer (LSRFortessa, BD Biosciences, USA). Stained DNA/RNA was excited via 488 nm blue laser and emission detected for total nucleotide detection (Alexa Fluor 488 filters, Life Technologies). Detection of valid signals was a combined signal of forward- and side-scattering and both fluorescence signal of SYBR Gold and chlorophyll autofluorescence. FACS data were analyzed by FlowJo 10 flow cytometry analysis software (FlowJo LLC, USA).

5.3.2. _Exaiptasia_ and coral cultures

Anemones of the single, clonal genotype _Exaiptasia_ (Grajales and Rodriguez, 2014) strain CC7 in association with _Symbiodinium_ strain SSB01 (_Symbiodinium minutum_) were generated and reared as described previously (Baumgarten et al., 2015). Anemones were cultured under a 12 h/12 h light/dark cycle under a photonflux of 20-40 µmol m$^{-2}$ s$^{-1}$ at 25°C and fed twice a week with _Artemia_. To avoid _Artemia_ contamination, food supply
was ceased five days prior to experiments. For experimental treatments, a six-well cell culture plate was prepared with 6 mL autoclaved Red Sea water (salinity: 38) per well. Seawater in three wells was adjusted to salinity of 35 and to 42 in the other three wells by addition of ddH$_2$O and NaCl, respectively. One *Exaiptasia* polyp was transferred into each well (i.e., triplicate samples for both salinities and one polyp for each sample and extraction were used) and kept at a 12 h/12 h light/dark cycle at 25°C for 15 h. Subsequently, each *Exaiptasia* colony was transferred into a 1.5 mL cryotube, rinsed thrice with filtered seawater, and frozen and stored in liquid nitrogen.

Corals were kept in long-term culture (>24 months) in different compartments of the experimental coral mesocosm facility at the University of Southampton at salinity levels matching those of their habitats of origin (salinity 42, *P. lobata*, Persian/Arabian Gulf (D’Angelo et al., 2015), salinity ~36.5, *P. lichen*, *H. grandis*, Indopacific (D’Angelo et al., 2008; D’Angelo and Wiedenmann, 2012; D’Angelo et al., 2015). *P. lobata* were additionally cultured under reduced salinity conditions of 34 for >24 months. Corals were kept at a temperature of 26°C with a 10 h/14h light/dark cycle under a photonflux of 150 μmol m$^{-2}$ s$^{-1}$ (D’Angelo et al., 2015). Light and temperature levels suitable for long-term culture of the corals were established during previous work (D’Angelo et al., 2008; D’Angelo and Wiedenmann, 2012). These three species were studied owing to their different capacity for survival at elevated salinities: *Porites lichen* associated with *Symbiodinium* sp. type C96 (short-term survival, (D’Angelo et al., 2015)), *P. lobata* with *S. thermophilum* (long-term survival (D’Angelo et al., 2015; Hume et al., 2016)), and *Hydnophora grandis* with *Symbiodinium* sp. type C40 (long-term survival). In the latter case, survival capacity was determined by incubating ten replicate colonies >24 months at a salinity of 42. In contrast to other Indopacific species (D’Angelo et al., 2015), these corals have not suffered any mortality and have been actively growing during this time (D’Angelo and Wiedenmann,
In the present experiments, replicate colonies of *P. lichen* and *H. grandis* previously cultured at lower salinities (34 and 36.5) were gradually adjusted to the high salinity of 42 over two days before being moved to the high salinity compartment for 12 days prior to sampling. All replicate colonies were produced by earlier fragmentation. Using an airbrush, coral tissue was blasted off the skeleton with ice-cold sterile-filtered freshly prepared artificial seawater with the same salinity as the culture rearing water. Three coral colonies were used per species to extract *Symbiodinium* cells. Algal cells were precipitated from the homogenate by centrifugation at 2,500 x g for 5 min at 4°C. Cells were washed twice (to remove the host tissue fraction and to prevent residual salt to interfere with the downstream GC-MS analyses), first in precooled seawater, then in ddH₂O, each step followed by centrifugation and re-suspension. The exposure to ddH₂O was limited to ~30 s to minimize potential effects of the hypoosmotic environment. After the final centrifugation step, all liquid was removed and the cell pellet was lyophilized for 14 h.

5.3.2. Metabolite extraction and recovery

Cell pellets from *Symbiodinium* cultures, *Symbiodinium* extracted from coral tissues, and whole-animal symbiotic *Exaiptasia* (due to low biomass) were resuspended and washed with 30 mL sterile seawater on ice, pelleted, and washed for ~30 s with further 5 mL ddH₂O to remove residual salt. After a further centrifugation step, pellets were re-suspended in 5 mL ddH₂O and cells disrupted by tip-ultrasonication for 4 min at 3 s pulsing, 6 s pause. Cell debris was removed by centrifugation at 20,000 g for 20 min at 4°C. Proteins/DNA/RNA were removed by ethanol precipitation by adding 9 parts of -20°C ethanol to 1 part supernatant. The precipitate was pelleted and removed by centrifugation, while the supernatant was frozen in liquid nitrogen and lyophilized. Dry samples were dissolved in 240 µL ddH₂O water, spiked with 10 µL of internal standard (1
µg/µL hydroxy benzylic acid (HBA) in ddH2O), transferred into GC vials, and dried under vacuum. For derivatization, 50 µL of MOX reagent (2 % methoxamine HCL in pyridine) was added to each sample and the solution was heated to 75°C for 1 h. Afterwards, 100 µL of MSTFA solution (MSTFA, 1 % TMCS, ThermoScientific) was added and samples were heated for 1 h at 75°C. Each sample vial was centrifuged at 2,000 x g for 10 min, and 100 µL of the supernatant was transferred to glass inserts placed inside GC vials.

5.3.3. GC/MS analysis and quantification

Derivatized carbohydrates, amino acids, and further intracellular compounds were characterized and quantified by GC-MS. For separation a HP-5ms column (Agilent Technologies, USA) and a temperature profile starting at 70°C was chosen. Temperature was increased by 6°C min⁻¹ up to 230°C, followed by 60°C min⁻¹ at a maximum of 280°C and held for 4 min. Metabolites were quantified by standard curves produced with pure glucose (99.5 %, Sigma, GER) and glycerol (ACS reagent ≥99.5 %, Sigma) with 60, 30, 10, 1 and 0.1 µg of both compounds. The calibration standards were spiked with 1 µg of HBA and derivatized for GC-MS and analyzed as described above. All samples were prepared and measured in triplicates. GC-MS data were processed (i.e., background subtraction, peak picking, and integration; OpenChrom v. 0.901, Lablicate UG, GER) and MS-ionisation spectra identified (NIST MS Software 2.0, Agilent Technologies). Statistical testing was conducted on normalized quantities of metabolites (nmol) using ANOVAs and Tukey's HSD post-hoc tests to assess differences between pairwise comparisons (for Exaiptasia at a salinity of 35, we only obtained 1 measurement, and hence, no statistical testing could be conducted). In the case of cultured Symbiodinium, we normalized to 100,000 cells mL⁻¹. For Exaiptasia, we used one animal of equal size and age per replicate and extraction.
Correspondingly, we measured total holobiont osmolyte and floridoside levels in *Exaiptasia*. For corals, *Symbiodinium* extracts were normalized over dry weights.

5.3.4. Floridoside homologs in cnidarian and *Symbiodinium* genomes

We searched for homologs of the putative enzyme that converts Glycerol-3-phosphate to floridoside Gasu_26940 (Pade et al., 2015) in the available symbiotic cnidarian and *Symbiodinium* genomes via BLASTp on reefgenomics.org (Liew et al., 2016) using an e-value cutoff of <10⁻⁵. Briefly, the amino acid sequence for Gasu_26940 was queried against the genomes of *Exaiptasia* (Baumgarten et al., 2015), *Acropora digitifera* (Shinzato et al., 2011), and *Stylophora pistillata* (Bhattacharya et al., 2016), as well as against the genomes of *S. microadriaticum* (Aranda et al., 2016), *S. minutum* (Shoguchi et al., 2013), and *S. kawagutii* (Lin et al., 2015).

5.4. Results

5.4.1. High levels of floridoside in *Symbiodinium* exposed to high salinities

First, we screened *Symbiodinium* cultures exposed to different salinities (i.e., low salinity: 25, ambient salinity: 38, high salinity: 55) for the presence of carbohydrate COOs. A markedly high abundance of a compound at 31.5 min retention time (RT) in the gas chromatography/mass spectrometry (GC-MS) trace was detected in all four tested *Symbiodinium* cultures under high salinity (i.e. *Symbiodinium microadriaticum* type A1, *Symbiodinium* sp. type A1, *Symbiodinium minutum* type B1, and *Symbiodinium psygmophilum* type B2) (Figure 10, Table 5). This compound was identified as the derivative of floridoside (2-O-glycerol-α-d-galactopyranoside-(hexa-TMS)) by a search against the National Institute of Standards and Technology Mass Spectrometry (NIST MS)
library with a reverse match factor of 971/1000 (Supplementary Table S3).

Figure 10. Gas chromatography analysis of carbohydrate osmolyte diversity in Symbiodinium psygmophilum at three salinity levels. Low salinity level of 25 (green), ambient salinity level of 38 (black), and high salinity level of 55 (red). The trace from 26 to 32 min retention time (RT) shows a floridoside peak at RT 31.5 min. The abundances are normalized to an internal standard (ISTD). The chemical reaction depicts synthesis of floridoside (3) as a product of uridine diphosphate (UDP)-galactose (1) and glycerol-3-phosphate (2). Of note, nearly identical traces were obtained for the other three Symbiodinium strains.
Table 5. Compatible organic osmolyte (COO) concentrations of four *Symbiodinium* strains at three salinities. Carbohydrates (floridoside, inositol, mannitol, glycerol, glucose, galactose, ribose, and fructose) and amino acids (glycine, alanine, valine, and proline) were quantified by GC-MS. COO levels are provided in nmol and normalized to GC-MS internal standard HBA and 1 x 10^5 cells ml^(-1) (i.e., concentration is nmol/10^5 cells or nmol/ml culture). *Symbiodinium microadriaticum* (type A1); *Symbiodinium* sp. type A1; *Symbiodinium minutum* (type B1); *Symbiodinium psygmophilum* (type B2); SE = standard error.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Salinity</th>
<th><em>Symbiodinium</em> microadriaticum</th>
<th>Symbiodinium sp. type A1</th>
<th>Symbiodinium minutum</th>
<th>Symbiodinium psygmophilum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. SE</td>
<td>Conc. SE</td>
<td>Conc. SE</td>
<td>Conc. SE</td>
<td>Conc. SE</td>
</tr>
<tr>
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<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
</tr>
<tr>
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<td></td>
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<td>1.2 0.2</td>
<td>3.7 0.5</td>
<td>19.6 2.5</td>
</tr>
<tr>
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<td>3.0 0.4</td>
<td>3.0 0.4</td>
<td>0.1 0.0</td>
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<tr>
<td>Glycerol</td>
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<td>92.9 11.7</td>
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<td>29.1 3.6</td>
</tr>
<tr>
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<td>106.3 13.4</td>
<td>21.9 2.9</td>
<td>12.3 1.6</td>
</tr>
<tr>
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<td>2.8 0.3</td>
<td>2.9 0.3</td>
</tr>
<tr>
<td>Fructose</td>
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<td>8.3 1.1</td>
<td>1.1 0.1</td>
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<tr>
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<tr>
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<td>30.0 3.8</td>
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<tr>
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<td>27.7 3.5</td>
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<td>Proline</td>
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<tr>
<td></td>
<td></td>
<td>12.2 0.8</td>
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<td>8.5 0.5</td>
<td>5.4 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.8 1.0</td>
<td>3.4 0.3</td>
<td>3.5 0.3</td>
<td>19.1 1.3</td>
</tr>
</tbody>
</table>
Floridoside concentrations of *Symbiodinium* strains exposed to high salinity ranged from 50 ± 7 nmol (*Symbiodinium minutum*) to 490 ± 55 nmol (*Symbiodinium psygmophilum*) and were consistently represented among the most abundant carbohydrates quantified in this analysis (Table 5). In contrast, floridoside was non-detectable under low salinity conditions for all *Symbiodinium* strains (Figure 11a, Table 5). At ambient salinity levels of 38, floridoside was only detected in *Symbiodinium psygmophilum* at a concentration of 51 ± 14 nmol (Figure 11a, Table 5). This strain also accounted for the highest measured amounts of floridoside under high salinity levels of 55 (490 ± 55 nmol). By comparison, inositol and mannitol were consistently present at low salinities and showed reduced concentrations at higher salinities for some strains (Table 5).

**Figure 11.** Floridoside levels of *Symbiodinium* in vitro and in hospite at different salinities. Floridoside levels represent measured concentrations (nmol) per 105 cells ml⁻¹ of algal cells (for cultured *Symbiodinium*), per dry weight (mg) of whole tissue homogenate (for *Exaiptasia*), and per dry weight (mg) of isolated *Symbiodinium* (for coral samples). Data obtained for each experiment were normalized to the highest value (set to 100%). (a) Floridoside levels in four cultured *Symbiodinium* strains in vitro at low (25), ambient (38), and high salinities (55). Smic: *Symbiodinium microadriaticum* (type A1), Sym A1: *Symbiodinium* sp. type A1, Smin: *Symbiodinium minutum* (type B1), Spsy: *Symbiodinium psygmophilum* (type B2). (b) Floridoside levels for the holobiont *Exaiptasia* (strain CC7) symbiotic with *Symbiodinium minutum* (strain SSB01) at salinities of 35 (1 replicate) and 42 (3 replicates) and for *Symbiodinium thermophilum* isolated from *Porites lobata* cultured at salinities of 34 and 42. (c) Floridoside levels of *Symbiodinium* from corals with a different long-term survival capacity at high salinities after incubation at a salinity of 42. Floridoside concentrations were determined for *Symbiodinium* sp. type C96 (*Porites lichen; short-term survival*), *S. thermophilum* (*P. lobata; long-term survival*), and *Symbiodinium* sp. type C40 (*Hydnophora grandis; long-term survival*). Error bars denote standard error (SE). Letters indicate Tukey’s HSD post-hoc test differences based on pairwise comparisons of ANOVA results (groups with different letters are significantly different at P<0.01 for (a) and P<0.05 for (c)).
We identified several other metabolites in the same GC-MS trace and could quantify a total of five additional carbohydrates (i.e., glycerol, glucose, galactose, ribose, and fructose) and four amino acids (i.e., glycine, alanine, valine, and proline) for each of our samples that serve as putative osmolytes. Concentrations of these metabolites were significantly different between different salinities (except proline) and *Symbiodinium* strains as well as combinations thereof (all *P*ANOVA*<*0.01) (Supplementary Table S4). This indicates that these metabolites are differentially regulated under changing salinities and in different *Symbiodinium* strains. Importantly though, only floridoside production showed a substantial increase at high salinities, whereas levels of all other metabolites (including mannitol and inositol) showed inconsistent patterns (Table 5).

Since floridoside can be derived from glycerol and glucose/galactose (Pade *et al.*, 2015), we investigated changes in the abundance of these molecules in detail (Figure 10, Figure 12, Table 5). In all *Symbiodinium* strains, a decrease in glycerol coincided with the accumulation of floridoside when comparing low to high salinity conditions (Figure 12, Table 5). Notably, glucose and galactose were enriched under high salinity conditions in *Symbiodinium microadriaticum*, *Symbiodinium* sp. type A1, and *Symbiodinium psygmophilum*; only in *Symbiodinium minutum* these sugars remained at the same level or showed a slight decrease between low and high salinity conditions (Figure 12, Table 5).
Figure 12. Osmolyte levels of floridoside and intermediates (glycerol, glucose, and galactose) at three salinities across four *Symbiodinium* strains. Glucose and galactose can be metabolized to glycerol (via the Calvin cycle) and cover UDP-galactose and glycerol-3-phosphate demands for floridoside synthesis. Floridoside, glycerol, glucose, and galactose levels for (a) *Symbiodinium microadriaticum* (type A1), (b) *Symbiodinium* sp. type A1, (c) *Symbiodinium minutum* (type B1), (d) *Symbiodinium psygmophilum* (type B2) cultures at low salinity (25, light gray), ambient salinity (38, gray), and high salinity (55, black) after 4 h at 108 μmol photons m$^{-2}$ s$^{-1}$. Letters indicate Tukey’s HSD post-hoc differences based on pairwise comparisons of ANOVA results (groups with different letters are significantly different at $P<0.05$). Tukey’s post-hoc tests were not performed if ANOVAs yielded a non-significant F-ratio, designated as n.s. (not significant).

5.4.2. High levels of floridoside in *Symbiodinium* from coral holobionts exposed to high salinities

To assess the importance of floridoside in holobionts, we exposed the coral model *Exaiptasia* associated with *Symbiodinium minutum* (strain SSB01) (Baumgarten *et al.*, 2015) and the coral *Porites lobata* associated with *Symbiodinium thermophilum* originating from the southern PAG (D’Angelo *et al.*, 2015) to different salinities and measured floridoside levels. All samples exposed to high salinity displayed a substantial
increase of floridoside (*Exaiptasia*: 94 % increase, *P. lobata*: 83 % increase) (Figure 11b, Supplementary Table S5).

We then compared algal floridoside levels in *Symbiodinium* from coral holobionts that show different capacities to survive at high salinity. We found that corals that were actively growing and surviving at a salinity of 42 for >24 months, i.e. *P. lobata* and *Hydnophora grandis*, had higher floridoside levels compared to those of *Porites lichen*, which is only capable to survive for a short period of time at this salinity (D’Angelo *et al.*, 2015) (Figure 11c, Supplementary Table S5).

We also checked for homologs of the putative enzyme that converts Glycerol-3-phosphate to floridoside in the available genomes of *Exaiptasia* and the corals *Acropora digitifera* and *Stylophora pistillata* to assess whether cnidarian hosts are in principle able to synthesize floridoside. Following (Pade *et al.*, 2015), we found no homologs for the gene that encodes the enzymatically active floridoside phosphate synthase/phosphatase in the red alga *Galdieria sulphuraria* (Gasu_26940) in the corals *Acropora digitifera* or *Stylophora pistillata* and only a partial hit in the genome of *Exaiptasia* CC7 (AIPGENE13675). Conversely, we found putative full-length homologs of the gene in all available *Symbiodinium* genomes, i.e. *Symbiodinium microadriaticum* (Smic14738, Smic32192, Smic6078), *Symbiodinium minutum* (symbB.v1.2.003359, symbB.v1.2.013114, symbB.v1.2.013196), and *Symbiodinium kawagutii* (Skav203497). Hence, the coral genomes investigated do not harbor the enzyme to produce floridoside. Although there is a possibility that *Exaiptaisa* is capable of producing floridoside based on a partial hit, this should be clarified in future experiments.
5.5. Discussion

In this study, we determined levels of the carbohydrates floridoside, inositol, and mannitol in response to high salinity in *Symbiodinium in vitro* and *in hospite* to assess the capacity of these COOs to fulfill a function in osmoadaptation to high salinities. Notably, other osmolytes that were not measured in our study, e.g. taurine, betaines, or DMSP (Pierce, 1982; Yancey *et al.*, 2010; Eierman and Hare, 2014), might also contribute to osmoadaptation of *Symbiodinium*. Consequently, our data should not be considered a complete assessment of all osmolytes in *Symbiodinium*. Importantly, however, we identified the osmolyte floridoside consistently and in increased amounts in *Symbiodinium* at high salinities. This shows that cultured *Symbiodinium* cells produce floridoside in response to salinity stress. Elevated floridoside levels were also evident in *Exaiptasia* and *P. lobata* when exposed to high salinity. In particular, we found elevated floridoside levels in *Symbiodinium* of corals capable of long-term survival at high salinity conditions (*P. lobata* and *H. grandis*). As such, our work has uncovered a key COO that we suggest allows *Symbiodinium* to osmoadapt to extreme salinities *in vitro* and *in hospite*. The fact that our results show a consisted accumulation of floridoside in response across a range of *Symbiodinium* strains and experimental conditions provides strong support for the notion that increased floridoside levels constitute part of the osmoadaptive response to high salinity. Our findings also provide insight for our understanding of the role of osmoadaptation in the coral-*Symbiodinium* endosymbiosis with implications for the coral stress response as further discussed below.

5.5.1. Floridoside as a key osmolyte in *Symbiodinium*

Synthesis of the osmolyte floridoside has been identified as a conserved pathway in evolutionary distinct organisms such as red algae, green algae, and cryptophycae.
It is produced by uridine diphosphate (UDP) galactosyltransferases via condensation of glycerol-3-phosphate and UDP-galactose (Pade et al., 2015). UDP-galactose demands can be supplied via starch mobilization resulting in increased glucose/galactose pools, as described for the green algae Dunaliella sp. under conditions of high salinity (Liska et al., 2004). A similar mechanism might explain the increased levels of glucose (in cultures of Symbiodinium microadriaticum and Symbiodinium sp. type A1) and galactose (in all cultured strains) that we measured in Symbiodinium at high salinity (Figure 12, Table 5). The consistent increase of glucose and galactose in concert with the upregulation of floridoside suggests that they fulfill a conserved osmotic adjustment function within the genus Symbiodinium (Figure 12, Table 5). Besides UDP-galactose, the second component required for floridoside synthesis, glycerol-3-phosphate is likely supplied from photosynthesis or via the Calvin cycle (Ben-Amotz and Grunwald, 1981). Glycerol-3-phosphate can be produced from glycerol, which is considered to be one of the main COOs in marine algae (Mayfield and Gates, 2007), although it has been shown to be released under osmotic pressure in Symbiodinium (Burriesci et al., 2012; Suescún-Bolívar et al., 2012; Suescún-Bolívar et al., 2016). Even though our preliminary analysis on available Symbiodinium genomes confirmed the presence of homologs for the enzyme that produces floridoside, it will be interesting to check for the presence and identity of the enzymes required for floridoside synthesis in ecologically relevant Symbiodinium (e.g., Symbiodinium thermophilum), and whether gene expression or duplication can be aligned with strain- or species-specific differences (Aranda et al., 2016).
5.5.2. Osmoadaptation in the coral-*Symbiodinium* endosymbiosis with implications for the coral stress response

Our results demonstrate that exposure to high salinities leads to higher endosymbiont floridoside levels *in vitro* and *in hospite*. This may point to that increasing floridoside levels not only increase the capacity of *Symbiodinium* to cope with the effect of osmotic stress in extreme environments, but also the capacity of the holobiont. Further, our results suggest that osmolarity changes within the coral tissue are noticed by endosymbiotic *Symbiodinium*, and thus, both the coral and *Symbiodinium* respond to salinity changes, presumably by adjusting the inner osmolarity to the higher outside salinity. It remains to be determined, however, whether *Symbiodinium* adjust their inner osmolarity the same way *in vitro* as *in hospite* (Yancey *et al.*, 2010). Importantly, potentially any metabolite contributes to the osmolarity, and thus the endosymbiotic environment in coral cells might differ from the seawater environment (Mayfield and Gates, 2007).

Beyond its function as an osmolyte, floridoside has been shown to act as an antioxidant with ROS scavenging properties (Li *et al.*, 2010; Kim *et al.*, 2013). As such, floridoside has the capability to convey osmoadaptation as well as to counter ROS produced in response to the salinity stress (Hasegawa *et al.*, 2000; Li *et al.*, 2010; Bose *et al.*, 2014; Hoef-Emden, 2014). In particular, increased ROS detrimentally affects photosystem II in photosynthetic organisms (Lesser, 2006; Murata *et al.*, 2007; Latifi *et al.*, 2009; Lesser, 2011). Therefore, the production of antioxidants during salinity stress is potentially important for *Symbiodinium*, and floridoside represents an osmolyte that fulfills a ROS scavenging function at the same time (Li *et al.*, 2010). Increasing levels of floridoside and oxidative stress in response to increased salinities were shown in the red algae *Gracilaria sordida* (Ekman *et al.*, 1991) and *Gracilaria corticata* (Kumar *et al.*, 2010), respectively. Future work should determine the exact role that floridoside plays in the salinity stress response,
either as an antioxidant, as a COO, or both, e.g. by comparing floridoside and ROS levels at ambient and increased salinities.

The notion that ROS producing mechanisms for photosynthetic organisms are similar (if not the same) under salinity and heat stress (Murata et al., 2007) has also interesting implications for our understanding of the response to heat stress in Symbiodinium, and by extension, for the coral hosts. Similar to salt stress, heat stress results in a malfunction of the photosynthetic machinery of Symbiodinium and the production of ROS that may damage the algal cells and, in the case of the coral-algal endosymbiosis, may trigger bleaching (Lesser, 1997). We find that Symbiodinium exposed to high salinity in vitro and in hospite accumulate high amounts of floridoside. As such, the production of floridoside in high salinity environments may thus increase the ability to sustain heat stress in Symbiodinium and, by extension, their coral hosts through scavenging of increased ROS levels by high levels of floridoside. Consequently, the thermal resilience of coral holobionts may potentially increase under conditions of high salinity due to the accumulation and inherent antioxidative capabilities of floridoside. Experimental data connecting increased floridoside levels to decreased ROS and bleaching levels under increased salinities are in demand to support this potential link.

5.6. Author contributions & Acknowledgements

General: We thank N.M. Kharbatia and A. Ortega for assistance in method development, measurements, and analysis; M. Ziegler and A. Roik for support in statistical analysis; X. Gong for Symbiodinium extraction; T. LaJeunesse for strain determination; H. Gegner for preparation of Aiptasia anemones.
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**Author contributions:** MAO, TR, CRV designed and conceived the experiments. MAO, TR, CRV generated, analyzed, and interpreted data. CRV, CDA and JW contributed cultures/reagents/materials. CRV, TR, CDA, JW, and MAO wrote the manuscript.

5.7. **References**


Lajeunesse, T.C., Parkinson, J.E., and Reimer, J.D. (2012). A genetics-based description of *Symbiodinium minutum* sp. nov. and *S. psygmophilum* sp. nov. (Dinophyceae), two dinoflagellates symbiotic with cnidaria. *Journal of Phycology* 48, 1380-1391.


5.8. Supplementary Material

Supplementary Table S3. Metabolites in GC/MS traces and Identification information for NIST MS 2.0 Library Search. Match factors are N of 1000. Detailed hits denote the primary search matches regarding ionization pattern match factors. RT: retention time.

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<th>Match Factor</th>
<th>Reverse Match Factor</th>
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<td>9.549</td>
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<tr>
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<td>881</td>
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Supplementary Table S4. Statistical evaluation of metabolite concentration changes of carbohydrates and amino acids across four Symbiodinium strains at three salinities. 2-Way ANOVAs, Symbiodinium strain and salinity level as fixed factors, strain*salinity as interaction effect, significance levels $P \leq 0.01$ in bold.

<table>
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<tr>
<th>Measured Metabolite</th>
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<th>Salinity</th>
<th>Strain*Salinity</th>
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<tr>
<td></td>
<td>$F$</td>
<td>$P$</td>
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<tr>
<td>Floridoside</td>
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<td>&lt;0.0001</td>
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<td></td>
<td>&lt;0.0001</td>
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<td>&lt;0.0001</td>
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</tr>
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<td>&lt;0.0001</td>
</tr>
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<td>Proline</td>
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Supplementary Table S5. Overview over floridoside amounts in cnidarian holobionts at different salinities. Values were normalized to ITSD and dry weight measures or animal counts. TIC = total ion count; SE = standard error; ND = not determined.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Salinity</th>
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<th>SE</th>
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</thead>
<tbody>
<tr>
<td><em>Exaiptasia</em> (<em>S. minutum</em> SSB01)</td>
<td>35</td>
<td>157,932</td>
<td>ND</td>
</tr>
<tr>
<td><em>Exaiptasia</em> (<em>S. minutum</em> SSB01)</td>
<td>42</td>
<td>2,657,253</td>
<td>69,833</td>
</tr>
<tr>
<td><em>Porites lobata</em> (<em>S. thermophilum</em>)</td>
<td>34</td>
<td>1,110</td>
<td>444</td>
</tr>
<tr>
<td><em>Porites lobata</em> (<em>S. thermophilum</em>)</td>
<td>42</td>
<td>6,689</td>
<td>3,583</td>
</tr>
<tr>
<td><em>Hydnophora grandis</em> (<em>Symbiodinium</em> sp. clade C40)</td>
<td>42</td>
<td>17,708</td>
<td>3,565</td>
</tr>
<tr>
<td><em>Porites lichen</em> (<em>Symbiodinium</em> sp. clade C96)</td>
<td>42</td>
<td>2,224</td>
<td>930</td>
</tr>
</tbody>
</table>
6. SYNTHESIS

Osmoregulation in corals is currently poorly understood. Periodical exposure to high salinities (e.g. in tidal rock pools or lagoonal areas with little water exchange) occurs regularly, but affects comparably few corals. In contrast, seasonal exposure to low salinities is more widespread, especially in high precipitation areas, and can lead to bleaching and mortality (True, 2012). Anthropogenic activities also influence salinity levels. On a regional scale an increasing fresh water demand in arid and semi-arid regions has led to a multiplication of desalination plants (Schiermeier, 2008; Elimelech and Phillip, 2011). These plants discharge high saline brine into the marine environment, including coral reef habitats. Global climate change is expected to lead to increased salinities in low precipitation areas and decreased salinities in high precipitation areas (IPCC, 2014). To date it is not clear how these salinity changes may affect corals, and the mechanisms involved in potential osmoregulatory processes are poorly understood in corals. Some coral habitats, for instance in the Red Sea and the Persian/Arabian Gulf (PAG), exhibit salinities considerably higher than in other regions which strongly suggests acclimatization/adaptation mechanism in corals (see 1.5).

This dissertation set out to assess the osmoregulatory capabilities of corals and to identify possible mechanisms involved. Briefly, all main compartments of the coral holobiont (i.e. coral host, algal symbiont, and associated bacterial microbiome) were addressed in their response upon short- (4 h and 15 h) and long-term salinity changes (7 d, 29 d, and >24 months). In this context, Fungia granulosa from the Red Sea, cultured Symbiodinium, the coral model Aiptasia, and corals from the PAG and the Indo-Pacific were assessed in a series of high salinity exposure experiments. Corals from the Red Sea and PAG provide an exceptional opportunity to study osmoregulation at the upper salinity limits of corals. This work further took advantage of a seawater reverse osmosis desalination plant.
(SWRO) to investigate its impact on corals. Similarly, the effect of salinity changes on *Symbiodinium in vitro* growth rates and putative osmolyte production was determined. Candidate osmolytes were validated in *Aiptasia* and corals from the PAG.

In the first chapter, data on salinity tolerance of the Red Sea coral *Fungia granulosa* were collected in a 29-day *in situ* transect study at a SWRO discharge site. To characterize the discharge dilution pattern, physico-chemical parameters salinity, temperature, and dissolved oxygen were measured. Considering the large amount (>40,000 m$^3$d$^{-1}$) and high salinity (>60) the dense brine discharged from the SWRO was expected to sink (Del Bene *et al.*, 1994). However, the lack of significantly increased salinity at the seafloor indicates a quick dilution of the SWRO brine. Owing to the quick dilution of the high saline brine, only corals positioned directly at the discharge screen were exposed to salinity increased by ~20% (from ~41 to ~49). Importantly, this chapter identified a remarkable long-term (29 d) salinity tolerance of *F. granulosa*. Photosynthetic performance of all corals remained unaffected during the experiment with photosynthetic efficiencies ranging around levels measured in *F. granulosa* in their natural habitat. Functional and stable photosynthetic performance under strongly increased salinity for 29 days indicates acclimatization and strongly suggests osmoregulatory processes within the coral holobiont. Most marine invertebrates, including sea anemones, are considered stenohaline osmoconformers with osmolyte levels in balance with the direct environment (Shick, 1991; Ferrier-Pages *et al.*, 1999; Kerswell and Jones, 2003; Hédouin *et al.*, 2015).

The here presented data strongly argue for the corals’ ability to actively adjust their osmotic equilibrium to pronounced salinity differences. Active osmoregulation generally requires energy, for instance for the active retention of water against an osmotic gradient and/or for the accumulation/synthesis of osmolytes. This increased energy demand may lead to long-term effects for the coral holobiont. Considering the large biomass of the
solitary coral *F. granulosa*, this coral species may be able to cover increased energetic demands for extended periods. The high salinity resilience and acclimatization pattern in *F. granulosa* only in part aligns with the literature. In other corals photosynthetic performance was consistently affected by moderate and strong salinity increases (Muthiga and Szmant, 1987; Manzello and Lirman, 2003; Lirman and Manzello, 2009). Yet, observed recovery under high salinity has been suggested as an acclimatization process (Manzello and Lirman, 2003; Lirman and Manzello, 2009).

In order to disentangle compartment effects on the long-term salinity resilience of coral host and algal symbiont, isolated *Symbiodinium microadriaticum* cultures were exposed to a series of different salinities between 25 and 55. All cultures displayed cell proliferation confirming an osmoregulatory potential in *Symbiodinium* (Goiran et al., 1997). Under salinity stress *Symbiodinium* likely requires an increased energy demand for active osmoregulation, as previously argued for *F. granulosa*. For *Symbiodinium*, this is reflected by decreased cell growth after seven days under all altered salinities compared to the controls. However, based on the growth of *Symbiodinium* in such a wide range of salinities, the dinoflagellate seems unlikely to limit the salinity acclimatization potential of the coral holobiont under naturally realistic scenarios. Yet, other *Symbiodinium* strains may differ in their physiological reaction and resilience to salinity variations.

To further investigate compartment and temporal effects on the osmoregulatory capabilities of *F. granulosa*, chapter 2 assessed the corals physiological response to short-term hypersalinity exposure and the response of the associated bacterial community to short- and long-term increased salinities. Short-term incubation experiments revealed an
increased mucus production and significantly impaired coral holobiont functioning. The produced mucus may act like a protective and/or physical barrier, likely restricting salinity exposure (Brown and Bythell, 2005). Nevertheless, short-term salinity exposure impacted the physiology of *F. granulosa*, resulting in decreased photosynthetic efficiency of the algal compartment and a stagnation in calcification processes of the coral host. The results differ from the findings in chapter 1, where unchanged photosynthetic performance and no mucus production were observed between 24 h to 29 d exposure to hypersalinity. This pronounced response to a sudden and strong increase in salinity may indicate the onset of osmoadjustive mechanisms preceding an acclimatization process, as suggested for other corals (Manzello and Lirman, 2003; Chartrand et al., 2009; Lirman and Manzello, 2009).

The third compartment of the holobiont, the associated microbial community, are thought to influence a coral’s ability to acclimatize and/or adapt to new environmental conditions (Reshef et al., 2006). Microbial communities and especially bacteria play a role in coral holobiont health, fitness, and nutrient acquisition and cycling (Rosenberg et al., 2007). In this regard, the second chapter focused on dynamics and putative functional implications of coral associated bacterial communities after short- and long-term exposure to increased salinities. The bacterial community composition in *F. granulosa* tissue from specimens collected in the reef and after short- and long-term exposure to hypersalinity were determined and compared. According to what is commonly found (Rohwer et al., 2002; Ziegler et al., 2016), all coral associated bacterial communities were distinct from the surrounding water, indicating that the coral hosts maintain bacterial associates distinct from their environment. Short-term exposure to elevated salinities did not result in distinct changes compared to freshly collected corals, which might be explained by bacterial doubling times of 10+ hours in corals (Apprill et al., 2009). However, after 29
days under hypersalinity the coral associated bacterial assemblages changed significantly. The coral bacterial microbiome after a long-term hypersalinity exposure further corroborates acclimatization of the coral holobiont. The maintenance of a heterogeneous and selected microbiome that is distinct from the surrounding water suggests the corals to be functional largely unimpaired. Further, the assemblages did not contain high numbers of pathogens and the numerically most abundant taxa hold the potential for beneficial functions. In this context, an unclassified member of the family Rhodobacteraceae was replaced by *Pseudomonas veronii* as the most abundant taxon. Rhodobacteraceae are a functionally diverse family, including denitrifiers (Neulinger *et al.*, 2008) and oil degraders (Kostka *et al.*, 2011). Similarly, *P. veronii*, is a versatile taxon found in fresh spring water and contaminated environments (Elomari *et al.*, 1996; Onaca *et al.*, 2007). Recently *P. veronii* has been identified in different cnidarian holobionts from comparably high saline habitats, including *Porites lobata* from the Red Sea and the PAG (Hadaidi *et al.*, 2017), three deep sea coral species from the Red Sea (Röthig *et al.*, under revision), and the coral model *Aiptasia* reared in Red Sea water (Röthig *et al.*, 2016). The repeated and oftentimes numerically dominant occurrence of *P. veronii* suggests an important functional role within cnidarian holobionts, in particular under increased salinities in the Red Sea and the PAG. In this context the potential of *P. veronii* to produce polyhydroxybutyrate (PHB) may be significant. Besides the osmolytic function of PHB (Arora *et al.*, 2006; Soto *et al.*, 2012) it has also been shown to possess anti-adhesive activity against prominent vibrio pathogens in shrimp aquaculture (Kiran *et al.*, 2014). This at least speculatively provides a mechanism for microbial pathogen control.

A putative functional profiling also suggested the bacterial changes to benefit the holobiont. Distinct functional patterns in corals exposed to long-term hypersalinity changed compared to all other corals. The changes included an increase in nitrogen
cycling, potentially covering increased nutrient demands of the coral holobiont. Further, the storage of the osmolyte PHB (likely at least partly produced by *P. veronii*, see above) was enriched. Lastly, changes in the sulfur metabolism presumably resulted in an increased concentration of the antioxidant dimethylsulphoxide (DMSO), which can protect the photosynthetic apparatus from oxidative stress (*Sunda et al.*, 2002). The putatively beneficial changes in the microbiome strongly suggest a functional support for the coral holobiont during salinity acclimatization. However, it remains to be determined if bacterial composition changes are a passive process governed by environmental change (i.e. increased salinity) or based on active host selection for a functionally more advantageous microbiome. Especially in niche or extreme environments microbiomes seem to be able to support their hosts. For example, bacterial assemblages enriched in temperature stressed corals have been suggested to elevate the bleaching threshold of their hosts (*Ziegler et al.*, 2017b). Further, increasing bacterial diversity with depth may support the host in nutrient acquisition and recycling in the lower light habitat (*Hernandez-Agreda et al.*, 2016), which may even be more important in azooxanthellate deep-sea corals (*Neulinger et al.*, 2008). Yet, the tremendous diversity and complexity of the microbial networks remain to be a major challenge for research on coral holobiont functioning. Therefore, model systems have recently gained popularity. *Aiptasia*, the model organism for cnidarian-*Symbiodinium* symbiosis, has also been suggested for as a model for manipulative microbiome studies (*Röthig et al.*, 2016). In parallel to work in other models (*Fraune et al.*, 2015), this system may allow testing for specific changes induced by isolated microbial members on holobiont function.
The first two chapters of this thesis identified osmoacclimatization in the coral holobiont, characterized the osmoadjustive capabilities of *Symbiodinium* in culture, and addressed the response of the associated bacterial microbiome upon salinity acclimatization. To determine potential osmoregulatory mechanisms in the coral holobiont, the exchange of metabolites between all compartments (i.e. coral host, *Symbiodinium*, and associated microbiota) must be considered. The coral host must equilibrate external osmotic pressure with its intracellular environment, which is determined by its own metabolism and that of its algal (and other microbial) symbionts (Mayfield and Gates, 2007). The synthesis/accumulation of osmolytes such as carbohydrates and amino acids is a widespread mechanism to maintain osmotic equilibrium under increased salinities in unicellular algae (Kirst, 1990) and in sea anemones (Shick, 1991). In corals carbohydrates that are transferred from *Symbiodinium* to the host (Muscatine, 1967) are thought to provide osmolytic functions (Mayfield and Gates, 2007). Accordingly, the third chapter assessed potential osmolytes involved in the osmoadjustment of the coral holobiont with the focus on *Symbiodinium*. Upon salinity change concentrations of four amino acids (i.e. glycine, alanine, valine, and proline) and eight carbohydrates (i.e. floridoside, inositol, mannitol, glycerol, glucose, galactose, ribose, and fructose) were determined in four *Symbiodinium* strains. Interestingly, unlike the other measured metabolites, the carbohydrate floridoside was consistently absent in decreased salinity. At ambient salinity floridoside was only present in one *Symbiodinium* strain, albeit at low concentration. Most importantly, under hypersalinity floridoside was consistently present in all strains at high concentrations (compared to all other measured metabolites) suggesting a pronounced and conserved role in the osmoadjustment of *Symbiodinium* under increased salinity. Floridoside is an important osmolyte in several evolutionary distinct organisms such as red algae, green algae, and cryptophycae (Eggert and Karsten,
2010; Hoef-Emden, 2014; Pade et al., 2015) but has not yet been described in *Symbiodinium*. It is synthesized from glucose/galactose and glycerol, a common osmolyte in unicellular algae (Pade et al., 2015). In all *Symbiodinium* strains glycerol concentrations decreased at hypersalinity compared to ambient conditions, presumably for the synthesis of floridoside. However, increased osmotic pressure also stimulates glycerol release from the algal cells (Suécün-Bolívar et al., 2012; Suécün-Bolívar et al., 2016). Glycerol transferred from the algal cells may support the host to establish an osmotic equilibrium, however further research is required to unequivocally identify the fate of glycerol. All other measured metabolite concentrations also changed upon salinity treatment albeit displaying inconclusive patterns that suggest only minor roles in the corals’ osmoregulation.

Upon identification of the osmolyte floridoside in *Symbiodinium* cultures, this chapter further validated the functional importance of floridoside in the holobiont framework. To do so, floridoside levels were assessed in a suite of cnidarian holobionts exposed to different salinities. In the coral model *Aiptasia*, floridoside levels strongly increased upon short-term exposure to hypersalinity. Similarly, floridoside levels in *Symbiodinium* extracted from the PAG coral *Porites lobata* were strongly increased in specimens long-term reared under high salinity compared to the controls from ambient salinity. To investigate the role of floridoside upon survival capacity of coral holobionts in high salinities, floridoside level in three coral species with different salinity tolerance thresholds were determined. The corals *Porites lichen* (from the Indo-Pacific), *Porites lobata* (from the PAG), and *Hydnophora grandis* (from the Indo-Pacific) differ in their associated *Symbiodinium* clades and in their capacity to survive increased salinities (D'Angelo et al., 2015; Hume et al., 2016). Importantly, upon exposure to high salinity (42) for 12 days corals with generally higher survival capacity in respect to increased salinities
contained higher floridoside level \((P. lichen < P. lobata < H. grandis)\). Taken together, floridoside levels after short-and long-term salinity exposure further point towards its fundamental and conserved osmoregulatory role in cnidarian holobionts.

The three chapters of this thesis suggest that all compartments of the holobiont contribute to salinity adjustment. Based on the cessation of calcification in the short-term incubation, the coral host is able to sense changes in external osmotic pressure. Similarly, Symbiodinium \textit{in vitro} responds to short-term salinity increase by accumulation of floridoside demonstrating its ability to sense changes in osmotic pressure inside the host cells. The accumulation of osmolytes further indicates \textit{Symbiodinium} to be an osmoconformer establishing an internal and external osmotic equilibrium. In line with the culture experiments, \textit{Symbiodinium in hospite} is also able to sense osmotic changes and synthesize floridoside indicating a change of the host’s internal osmotic pressure. Based on the glycerol decrease in cultured \textit{Symbiodinium} and according to Suescún-Bólivar \textit{et al.}, (2016), the osmolyte glycerol can be transferred to the host where it may be employed as an osmolyte further contributing to the coral holobionts salinity response. The contribution likely varies between different \textit{Symbiodinium} strains based on the heterogeneous floridoside levels measured in different symbionts. This is in line with previous findings where different \textit{Symbiodinium} types varied in their performance under and resilience towards environmental stress. For instance, \textit{Symbiodinium} from clade D are generally believed to be thermotolerant (Berkelmans and van Oppen, 2006; Oliver and Palumbi, 2011), and a high resilience in certain clade C types has also been reported (Abrego \textit{et al.}, 2008; Hume \textit{et al.}, 2015). It seems clear, that specific \textit{Symbiodinium} strains can influence the geographical distribution of their respective hosts, also in terms of heat.
and salinity thresholds (D’Angelo et al., 2015; Hume et al., 2015; Ziegler et al., 2015, Ziegler et al., 2017a). Unlike for the dinoflagellate symbiont, this thesis does not provide detailed information on mechanistic osmotic adjustment of the coral host itself. It seems likely though, that the coral host is not able to synthesize floridoside itself based on the lack of gene homologs encoding for enzyme involved in the synthesis. This suggests that different osmolytes may be prominent in the host cells, for example betaines and taurines (Yancey et al., 2002).

Further functional importance of floridoside in the osmoadjustment in Symbiodinium and in coral may arise from its antioxidative capacity with ROS scavenging properties (Li et al., 2010; Kim et al., 2013), thereby potentially providing an increased protection from ROS. As shown in plants and algae (Hasegawa et al., 2000; Bose et al., 2014), salinity stress is assumed to increase the production of harmful ROS within algal symbionts, which must be counteracted by the coral holobiont (Mayfield and Gates, 2007). Higher antioxidant levels result in an increase in salinity tolerance in plants (Hasegawa et al., 2000). Certain compounds like superoxide dismutase, glutathione, and DMSP and its breakdown products have been described as an antioxidative defense under environmental stress in coral (Lesser, 2006; Gardner et al., 2016) and under salinity stress in plants (Hasegawa et al., 2000). According to plants, in coral exposed to high salinity ROS scavenger - like floridoside - are likely involved, but have not been described yet. This work suggests that coral holobionts can further increase their salinity resilience by the accumulation of Symbiodinium produced floridoside and benefit from its dual function as an osmolyte and antioxidant. In line with this, dual mechanisms for coral osmolytes under ambient salinity have been suggested including thermostabilization (i.e., by glycine and betaine) and antioxidative properties (i.e., by DMSP) (Yancey et al., 2002). Similarly, floridoside may increase the resistance of the coral holobiont against additional environmental stress.
Accordingly, this thesis identified a potentially important, previously overlooked osmolyte with antioxidant properties.

In summary, this thesis provides strong arguments that corals can adjust osmolarity based on a series of short- and long-term experiments. It further strongly suggests that the associated bacterial community supports the acclimatization of the coral holobiont to increased salinity. The demonstrated osmoregulatory capabilities of the algal symbiont in culture corroborate the previously reported role of *Symbiodinium* in the coral holobionts ability to cope with different environmental conditions. The collected data indicate that upon salinity increase glycerol is transferred to the host and potentially supports the osmotic equilibrium of the coral holobiont. Importantly, at the same time floridoside synthesis within the algal cells increases enabling active osmoregulation in the algal cells. Floridoside’s dual function as an osmolyte and antioxidant potentially provides a mechanistic link providing protection form osmotic pressure and salinity induced ROS formation in symbiotic coral holobionts. In this context future research should validate an increased ROS production of corals exposed to increased salinity considering their floridoside levels. It would further be interesting to examine *in hospite* floridoside concentrations in different corals on a large scale to determine the prevalence and importance of the suggested mechanism. As a ROS scavenger, floridoside may also provide protection for the coral holobiont from other environmental stress. Importantly, this thesis shows that all compartments of the coral holobiont contribute to its salinity response and suggests that salinity is an important factor to consider for health and resilience of corals.
6.1. References


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7. APPENDIX

Supplementary File S1. Published article "High salinity tolerance of the Red Sea coral *Fungia granulosa* under desalination concentrate discharge conditions: An *in situ* photophysiology experiment".

Supplementary File S2. Published article "Long-term salinity tolerance is accompanied by major restructuring of the coral bacterial microbiome".

Supplementary File S3. OTU abundance over samples with annotation and reference OTU sequence. WS = water sample, FC = freshly collected corals, ST = short-term incubation; LT = long-term treatment; A = ambient; H = hypersaline; Numbers in the LT sample names denote transect station.

Supplementary File S4. OTUs enriched in fresh, short-term ambient and hypersaline, and long-term ambient corals (P ≤ 0.001, average abundance ≥10); OTUs enriched in long-term hypersaline corals (P ≤ 0.001, average abundance ≥10; abundance count in each of the three replicates).

Supplementary File S5. Submitted article “Osmolyte "moonlighting" renders symbiotic reef corals more tolerant to heat and salt stress".