# GLOBAL CHANGE STRESS ON SYMBIONT-BEARING BENTHIC FORAMINIFERA

**Dissertation**

**zur Erlangung des**

**Doktorgrades in den Naturwissenschaften (Dr. rer. nat.)**

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**Christiane Schmidt**

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**im Fachbereich Geowissenschaften**

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## Zusammenfassung

Symbionten-tragende benthische Foraminiferen sind wichtige Kalzit Produzenten in den Weltmeeren, welche zu einem Drittel an der Produktion des Karbonat-Sediments der Korallenriffgemeinschaften beteiligt sind. Die ansteigenden Meerwassertemperaturen verursacht durch den globalen Klimawandel, führen zu oxidativem Stress in den Symbiosen der Korallenrifforganismen, welche sehr temperatursensitiv sind. Das führt zur sogenannten „Korallen Bleiche“, welche auch Foraminiferen betrifft. Dieser Prozess ist gekennzeichnet durch den Farbverlust der Organismen, welcher mit einem Pigmentverlust einhergeht und zum Tod der Gemeinschaften führen kann. Weiterhin wird die Physiologie von Foraminiferen durch die Ozeanversauerung negativ beeinflusst. Dieser Prozess lässt den pH Wert des Meeres absinken und führt zu einer Abnahme der gelösten Carbonat-Ionen im Wasser, was entscheidend für Kalkbildungsprozesse der kalkschaligen Gehäuse ist.

In dieser Studie geht es um die gleichzeitigen Effekte von ansteigenden Meerwassertemperaturen und Ozeanversauerung auf Foraminiferen, welche in anderen Organismen gezeigt haben, dass sie sich gegenseitig verstärken. Frühere Studien an symbionten-tragenden Foraminiferen haben gezeigt, dass es artenspezifische Unterscheide in der Hitzetoleranz gibt. Das Ziel war es mehr über Hitzetoleranz in Foraminiferen zu erfahren, um diese besser in Bezug zu anderen Eukaryoten mit Photosymbiosen stellen zu können. Dazu haben wir an einer angeblich sehr hitze-stabilen Art *Pararotalia calcariformata* gearbeitet, welche lebend in einer thermalen Fahne eines Stromkraftwerks unter Temperaturen von 36°C im östlichen Mittelmeer an der Küste Israels gefunden wurde. Wir nutzten diese Art, um Temperaturstressexperimente im Bereich 20-42°C durchzuführen. Gleichzeitig exponierten wir eine zweite Art, *Amphistegina lobifera,* in Experimenten von 20-36°C, welche in der thermalen Fahne nicht beobachtet wurde, aber häufig an der Mittelmeerküste vorkommt. Wir führten die Experimente an natürlichen Populationen aus dem israelischen Nationalpark Nachsholim durch um zu testen ob *P. calcariformata* in einem Habitat ausserhalb der thermalen Fahne auch diese extreme Toleranz aufweist, oder ob sich dieses Phänomen auf die Population in der thermale Fahne beschränkt. Wir vermuteten, dass die ungewöhnlich gute Anpassung der Art an hohe Temperaturen auch mit deren geographischen Ursprung oder deren Einwanderung ins Mittelmeer zu tun haben könnte. Deshalb führten wir weitere ökologische, genetische und physiologische Studien an dieser Art und an ihren

Symbionten durch. Seit der Öffnung des Sueskanals sind viele Arten kürzlich ins Mittelmeer eingewandert und haben sich dort ausgebreitet, daher wollten wir auch testen ob sich die Verbreitung der Art *P. calcariformata* sich mit dem globalen Wandel nach Westen ausdehnen wird. Somit stellten wir experimentell fest, ob die Minimaltemperatur von 20°C, ein Hindernis für die Ausbreitung der Art darstellt, indem wir die Entwicklung von juvenilen Foraminiferen unter drei Temperaturen experimentell verfolgten.

Die Studie an den gleichzeitigen Effekten von Klimawandel und Ozeanversauerung zeigte, dass erhöhte Temperaturen die Arten *Heterostegina depressa* und *Marginopora vertebralis* negativ beeinflussen. Ergebnisse früherer Studien wurden somit bestätigt. Das zusätzliche Einwirken von Effekten der Ozeanversauerung, verstärkt den physiologische Stress auf die Arten und wirkt oft auch synergistisch. Weiterhin zeigen wir an Hand von Temperaturstressexperimenten, dass die Art *Amphistegina lobifera* ab einer Temperatur von 32°C photosynthetischem Stress ausgesetzt ist, und bestätigen damit frühere Studien aus Australien und Florida. Wir bestätigen für *P. calcariformata* eine extreme Hitzetoleranz für die Population aus dem israelischen Nationalpark Nachsholim in mehreren Laborexperimenten und zeigten damit, dass die Hitzetoleranz der Art nicht auf eine Hitzefahne eines Kraftwerks beschränkt ist. In der Population haben wir eine signifikante Reduktion der photosynthethischen Aktivität beginnend ab 36°C nach drei Wochen Exposition gemessen und permanente Photoinhibition bei 42° ab einer Woche. Wir zeigen, dass das Wachstum der Juvenilen am besten zwischen 24-28°C stattfindet und, dass es bei 20°C und 35°C inhibiert ist. Dies lässt vermuten, dass die Temperaturempfindlichkeit von *P. calcariformata* gegenüber niedrigen Temperaturen ein Grund ist, wieso sich die Art nicht bereits ins westliche Mittelmeer ausgebreitet hat, sondern bislang nur im östlichen Mittelmeer zu finden ist. Molekulare und taxonomische Analysen lassen weiterhin vermuten, dass die Art eine eingewanderte Art aus dem Indopazifik ist und dass der Temperaturanstieg im östlichen Mittelmeer, welcher auf den globalen Wandel zurückzuführen ist, entscheidend zur Ausbreitung der Art beigetragen hat.

Zusammenfassend zeigen wir, dass der globale Wandel die Physiologie von benthischen symbiont-tragenden Foraminiferen stärker beeinflusst, als die isolierten Effekte von Temperaturanstieg und Ozeanversauerung einzeln betrachtet. Wir

bestätigen artenspezifische Unterschiede in der Hitzetoleranz von symbionten-tragenden Foraminiferen. Weiterhin beschreiben wir die Physiologie von *P. calcariformata*, welche unter 36°C für mehrere Wochen photosynthetisch aktiv ist, und welche somit hitzetoleranter ist als die meisten Korallen und andere eukaryotischen Photosymbiosen. Die Ergebnisse lassen deuten, dass manche Arten bereits jetzt schon an höhere Temperaturen von 1-2°C über Sommermaxima adaptiert sind und somit besser mit den Folgen des Klimawandels umgehen können als andere Arten, welche unter diesen Bedingungen nicht existieren können.

## Summary

Symbiont-bearing benthic foraminifera are important calcite producers accounting for one third of the production of carbonate sediment in coral reef environments. Like other coral reef organisms with endosymbionts, they are sensitive to oxidative stress, induced by ongoing anthropogenic global climate change. Elevated temperatures affect the symbiotic relationship with marine microalgae, resulting in bleaching; defined as the loss of pigments from the host and eventually induce mortality. Additionally, foraminifera's physiology is negatively affected by ocean acidification, a process which results from increasing atmospheric carbon dioxide emissions, which lowers the pH of the ocean and reduces the availability of carbonate ions for the calcification processes of marine organisms.

This study uses foraminifera to establish whether elevated temperatures and ocean acidification acting in concert exaggerate the negative effects of these factors singularly, as shown in other studies. Previous studies on symbiont-bearing benthic foraminifera showed that there are species-specific differences in bleaching thresholds in foraminifera. To find out more about bleaching thresholds in foraminifera and to compare them to other eukaryotic symbioses, especially corals, we study an apparently very heat-tolerant foraminifer *Pararotalia calcariformata* which was recently observed to survive temperatures of 36°C inside a heat plume at a power plant in the eastern Mediterranean Sea, Israel. We conducted temperature exposure experiments in the range of 20-42°C on this species and in the range of 20-36°C on another abundant species *Amphistegina lobifera* in the Eastern Mediterranean which was not found to occur in the heat plume. We conducted the experiments on a population from outside of the heat plume to see if its unique thermal tolerance would be limited to the heat plume population or is also present in populations from thermally unpolluted habitat. As its apparently innate resistance to elevated temperatures could also have to do with the origin of *P. calcariformata* or its recent invasion in the Mediterranean, we conducted combined ecological, genetic and physiological studies on this species and its symbionts. As many species have recently invaded the Mediterranean and spread westwards with ongoing global warming, we wanted to test, if this is also likely for *P. calcariformata*. We also experimentally observed shell development and growth rates of juveniles under different temperatures to evaluate if the distribution of this species is constrained by colder temperatures to spread in the western Mediterranean.

The study on the combined effects of global warming and ocean acidification showed that temperature negatively affected *Heterostegina depressa* and *Marginopora vertebralis*, confirming previous results. In combination with ocean acidification the effects were stronger and often even synergistic. In temperature sensitivity experiments on *Amphistegina* we showed that its bleaching threshold is similar to earlier studies from Florida and Australia, and that temperatures above 32°C put stress on the photosynthetic activity of its symbiosis. For a *Pararotalia* population from a thermally unpolluted habitat a unique thermal tolerance was confirmed by laboratory experiments. This confirms that this thermal tolerance is not limited to the heat plume. We observed a significant reduction in photosynthetic activity first at 36°C after three weeks and chronic photoinhibition at 42°C after one week of exposure. We show that juvenile development is best between 24-28°C and inhibited at 20°C and 35°C, indicating that lower temperature in the western Mediterranean are a limiting factor for the establishment of new populations. Our molecular and taxonomic identification show that *Pararotalia* is a likely invader species from the Indo-pacific and that it could establish a recent population in the eastern Mediterranean Sea because of the ongoing warming trend. In conclusion we show that the combined effects of climate change and ocean acidification impact the physiology of symbiont-bearing foraminifera stronger than the individual effects and those are likely underestimated when stressors are evaluated in isolation. We confirmed species-specific differences in the thermal tolerance of symbiont-bearing foraminifera to the extent that we found an active photosymbiosis under 36° in the foraminifer *Pararotalia* for 3 weeks, which is higher than most corals symbioses and other eukaryote-eukaryote symbioses. Our results point out that some foraminiferal species seem to be well adapted to conditions 1-2°C above current summer maxima and they are likely to persist under global change conditions, where other species will not.

# 1. Introduction

## 1.1. The ecology of symbiont-bearing benthic foraminifera

Symbiont-bearing foraminifera are important producers of reef carbonate (Scoffin and Tudhope 1985; Langer et al. 1997; Doo et al. 2012). They act as ecosystem engineers (Langer et al. 2012; Weinmann et al. 2013) making up 30-90% of reef deposits in some Indo-pacific coral reefs as, for example, on Green Island, Australia (Fig. 1, 2 a, 2b) (Yamano et al. 2000) or Rain Island, Australia (Dawson et al. 2014). Along these islands empty shells are transported down slope and partly constitute coral sand, which help stabilize reef structures (Yamano et al. 2000; Hohenegger 2006). Stabilization of reef structures by calcium carbonate is crucial for coral reefs ecosystems, as it enables optimal light regimes for the different coral reef organisms (Liquete et al. 2013). A large fraction of the current carbonate production by larger benthic foraminifera (LBF) can only be achieved, because of the photo-symbiotic relationship with microalgae (Müller-Merz and Lee. 1976), which help them to obtain more energy for calcification (de Nooijer et al. 2009) in oligotrophic waters, and in return provide a protected and nutrient-enriched microenvironment for the symbionts (Lee and Hallock 1987). In addition to LBF stabilization and production of reef carbonate, the nocturnal dissolution of their empty Mg-calcite shells also acts as a buffer to daily pH changes in shallow reef environments, because dissolution of carbonates locally elevates alkalinity (Yamamoto et al. 2012).

In the tropical realm LBF inhabit the reef crest and slope and can be found until the end of the photic zone, as waters are usually more oligotrophic (Hohenegger 1994). In the tropics they live epiphytically on substrate which can be marine turf algae growing on dead coral reef rubble or stones (Nobes et al. 2008; Schmidt et al. 2011) and sea-grasses (*Marginopora vertebralis* from Chapter 1) (Fig.1, 2b). Individual species of LBF are distributed within a strict depth and habitat zonation reflecting light preferences and energetic water conditions (Hallock 1984; Baker et al. 2009). Outside of the tropics and in non-reef settings, they are found mainly on macro or turf algae growing epiphytically on bedrock environments or overhanging underwater cliffs. For example, they have been collected on filamentous coralline algae such as *Jania sp*. (*Amphistegina lobifera* studied in Chapter 2-4) (Fig. 1), or other seaweeds such as *Sargassum sp*. or *Cystoceira sp.* (Bresler and Yanko 1995).

It has been suggested that structural features in the habitat, nutritional and environmental gradients strongly shape biodiversity of epiphytic assemblages, as life spans of epiphytes might be dependent on seasonal availability of the substrate (Langer 1993). The substrate has multiple benefits for the LBF, it provides shelter against currents, it may provide additional food for LBF as they can graze on a surface layer of biofilms, and it enables the foraminifer to expose themselves optimally to the light and finally provides a nursing ground. Symbiont-bearing foraminifera are known to grow also without additional feeding, being nourished solely by their algal endosymbionts (Lee et al. 1991; Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014). Experiments showed that about 90% of the carbon requirements of the Soritid host can be covered through the symbionts (Lee and Bock 1976). Providing a mixture of living micro-algal diets isolated from natural habitat showed species-specific results (Lee et al. 1991) suggesting a high specificity in the host-symbiont relationship.

Foraminifera reproduce through a complex life cycle of asexual and sexual reproduction, varying a diploid (2n) agamont generation with a haploid (n) gamont generation (Grell 1973; Rottger 1974). The asexual mode of reproduction dominates the life cycle of LBF and facilitates the vertical transmission of the symbionts from mother to daughter cell (Pochon et al. 2006; Lee et al. 2009). When the mother organism undergoes asexual reproduction, multiple fission (meiosis) takes place dividing the cell and its nuclei to produce offspring with a haploid (n) set of chromosomes. The asexual juveniles usually leave the mother shell when they have calcified 2-4 chambers and may remain attached in brooding chambers at their adult individual before they are released in the sea water (Rottger 1974; Hohenegger 2011). Every young asexual gamont obtains the organelles and symbionts from the parent. Several hundred young gamonts have been observed to be released near simultaneously from the adult individuals (Hohenegger 2011). This form of reproduction is most frequently observed under culturing conditions (see Chapter 2), (e.g. Röttger and Berger 1972; Glas et al. 2012b; Hosono et al. 2014), indicating a higher success rate than sexual mode of reproduction.

In sexual reproduction, gametes (1-2µm size plus flagella) are released in the water, and fuse their nuclei by multiple fission and form a diploid (2n) zygote, from which a diploid (2n) agamont develops (Röttger et al. 1990; Dettmering et al. 1998).

This form has several disadvantages compared to asexual reproduction: gametes from different organisms need to be synchronically “spawned” in the water so that the chance of finding a partner is higher, especially as the survival time for the gametes is only a few days (Hohenegger 2011). As the diploid zygote does not contain symbionts the agamont must obtain new symbionts from the environment (Lee and Anderson 1991). In many foraminifera the agamont arising from the zygote has a smaller initial chamber (proloculus) than the gamont arising from the zygote (Grell 1973).


**Figure 1. Natural habitats of symbiont bearing benthic foraminifera from temperature and tropical locations A) *Heterostegina depressa* and B) several genus (*Calcarina, Bachologypsina*, *Operculina, Peneropolis*) from Orpheus Island, Australia, several scale bar: 1 mm, 2 A) Carbonate sediments and B) *Marginopora vertebralis* on sea grass at Green Island, 2 cm, 3 A,B, several Foraminifera on filamentous corralling algae *Jania sp.*, form Nachsholim Park, Israel, A) *Sorites* and B *Amphistegina lobifera* and *Textularia*, scale bar: 2 mm.**

## 1.2. The Identity and diversity of symbionts in benthic foraminifera

The symbiosis in benthic foraminifera is mutualistic, being beneficial for both partners (Lee and Hallock 1987). The symbionts are protected in the foraminiferal test, as “naked” cells, exposed to higher nutrient concentrations than when occurring free-living in the ocean. This is because the host facilitates internal recycling of nutrients, allowing high spatial density of the symbionts to exist inside the cell. In this way, the symbiosis represents an adaptation to oligotrophic conditions. Thus, photosymbiosis has been a driving force in foraminiferal evolution (Lee and Hallock 1987; Lee et al. 2010).

In comparison to other eukaryotes in shallow coastal seas, such as corals, sea anemones and giant clams which all host endosymbiotic dinoflagellates, LBF collectively host a broader taxonomic spectrum of endosymbionts including several microalgae and cyanobacteria (Lee and Anderson 1991). The microalgae diversity in LBF symbioses ranges from diatoms, dinoflagellates, red algae and green algae (Lee and Anderson 1991; Lee 2006). A single LBF genus is usually found to be associated with one type of microalgae at any one time (Lee 2006; Lee et al. 2010). The chambers of LBF are often highly compartmentalized and the compartments are connected by openings and foramina which allow the cell to distribute the symbionts in different zones (Müller-Merz and Lee. 1976; Lee and Anderson 1991). The symbionts are kept away from the digestive activities and are most densely concentrated around the inner zone (Fig. 2) (Müller-Merz and Lee. 1976; Fay et al. 2009). It has been shown that the “naked” endosymbionts have special surface antigens which prevent them to be digested by their host (Chai and Lee 2000).

Before molecular methods existed transmission electron microscopy was used to examine symbiont diversity in LBF (Müller-Merz and Lee. 1976; Gastrich 1987). The first morphological identification of the diatom symbionts outside of the host was possible as the “naked” endosymbiotic diatoms have been shown to form silica frustules when grown in antibiotic media. This approach has led to the characterization of the endosymbiotic diversity in diatom-bearing foraminifera (Lee et al. 1989). In total about 20 small diatoms (<10µm) have been found to be associated with LBF, most of them contain one or two, and occasionally a third species at any

one time. The most commonly observed symbiotic diatoms belong to the species *Nitzschia frustulum var. symbiotica* which has been isolated in one third of the investigated specimens (Lee et al. 1980; Lee 1991; Lee and Correia 2005). Other common species are *Nanofrustulum shiloi*, *Nitzschia laevis, Nitzschia panduriformis* and several species belonging to the genus *Amphora* (Lee and Correia 2005).

Furthermore, eight smaller foraminiferal genera such as *Elphidium* have been found to contain isolated diatom chloroplasts as symbionts, a phenomenon known as kleptoplastidy (Lee and Lee 1990; Bernhard and Bowser 1999; Pillet et al. 2011). The diatom organelles have a half-life of 9.5 weeks when cultured in the dark (Correia and Lee 2000; Correia and Lee 2002).

The disc-shaped foraminifera, for example the genus *Sorites*, *Amphisorus*, and *Marginopora,* contain endosymbiotic dinoflagellates of the genus *Symbiodinium* (Lee et al. 1997; Pawlowski et al. 2001). Genetic analysis of foraminiferal symbionts suggests that multiple *Symbiodinium* lineages uniquely associate with foraminifera (clades F3-F5, G1, H, I) (Garcia-Cuetos et al. 2005; Pochon et al. 2007) but dominant *Symbiodinium* types C3 and C15, which are common in corals (e.g. LaJeunesse et al. 2003; Cooper et al. 2011; Putnam et al. 2012) are also found in *Marginopora vertebralis* (Momigliano and Uthicke 2013). In comparison to most corals, the disk-shaped LBF host a more genetically diverse symbiont community (Pochon et al. 2007), suggesting that they may be a reservoir for *Symbiodinium* communities in the coral reef environment (Fay et al. 2009). The genus *Sorites* has been shown to also contain red cyanobacteria (Lee et al. 1997) or unicellular green algae *Chlamydomonas provasolii* (Müller-Merz and Lee. 1976).

Host-symbiont relationship in LBF seems to be flexible (Lee 2006; Momigliano and Uthicke 2013). It is not clear whether or not adult LBF can re-capture symbionts from the environment after symbiont-loss. An experiment by Lee et al. (1986) suggested that it is possible, however no symbionts could be isolated again from the re-browned hosts, suggesting that possibly symbionts where digested (Lee et al. 1986).

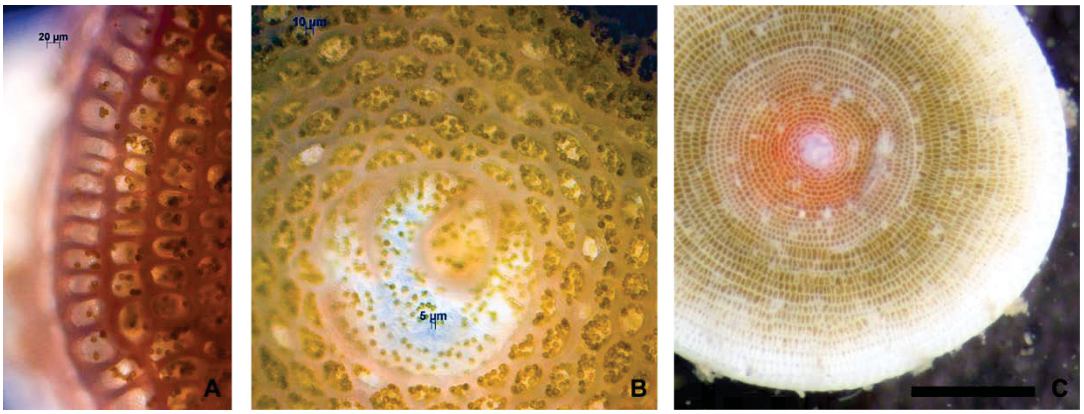


**Figure 2. Dinoflagellate symbionts in *Marginopora vertebralis* from Orpheus Island, Australia inside their host, A, B) Symbionts in light microscopy showing A) the edge B) proloculus area A) scale bar: 20µm, B) 5µm and 10µm, C) *Marginopora vertebralis* showing different coloration due to possibly different symbiont type or density in their shell, consisting of inner, middle and outer ring, scale bar: 2 mm.**

## 1.3. Bleaching in symbiont-bearing foraminifera

Bleaching, defined as the loss of microalgae or their associated pigments, has been documented in several symbiont associations with marine invertebrates, such as corals (e.g. Brown 1997; Berkelmans and Oliver 1999), crustose coralline algae (Anthony et al. 2008), sea anemones (Perez et al. 2001), sponges (Fromont and Garson 1999), giant clams (Addessi 2001) and recently also nudibranchs (Ziegler et al. 2014). The mechanisms of bleaching are currently not fully understood in LBF in comparison to corals (Brown 1997; Fitt et al. 2001). Bleaching was first described in benthic foraminifera in field populations in the 90's in the Florida Keys and the Bahamas (Hallock et al. 1992; Hallock and Talge 1993; Hallock et al. 2006). Bleached specimens of *Amphistegina* were observed as having color in only the last chambers or having mottled appearance (Fig. 3) (Hallock et al. 1992; Hallock and Talge 1993). Microscopic studies revealed ultra-structural damage when organisms experienced photo-oxidative stress (Talge et al. 1997; Talge and Hallock 2003). In corals it has been suggested that oxidative stress leads to coral bleaching, and can be induced by temperature increase or UV light. Corals have been shown to be resistant to bleaching and higher photosynthetic rates when they were exposed to antioxidants which hindered reactive oxygen species (ROS) formation (Lesser 1997). Oxidative stress, in any form, leads to the formation of reactive oxygen species (ROS) in the hosts tissues, because of higher metabolic rates (Sohal and Weindruch

1996). Reactive oxygen species are damaging to membrane function and in high concentrations may lead to bleaching and be lethal to the organisms (Sohal and Orr 2012). In the first laboratory experiment on bleaching in *Amphistegina gibbosa*, elevated temperature induced symbiont loss at 32°C, however at very low light intensities of 6-8 µmol photons $m^2$ $s^{-1}$, which might have additionally stressed the symbiosis (Talge and Hallock 2003). Similar levels of deterioration of the cytoplasm has been observed among field-stressed specimens of *Amphistegina gibbosa*, but also shell-breakage, symbiont-digestion, deformed tests and reproductive dysfunction, suggesting the possibility of other diseases affecting these populations (Talge et al. 1997) (Fig.3).

Physiological stress associated with elevated temperatures and subsequent bleaching was investigated using Pulse Amplitude Modulated (PAM) Fluorometry and photometric pigment measurements, which revealed that symbiont performance and chlorophyll a content was significantly reduced under temperatures >31°C in several LBF (Schmidt et al. 2011; van Dam et al. 2012a). Furthermore, these studies revealed species-specific results, as for example the species *Calcarina mayorii* did not show signs of stress under 32°C for 30 days (Schmidt et al. 2011). This suggests that responses do not generally depend on the microalgae type, as all species examined host endosymbiotic diatoms (Schmidt et al. 2011). Furthermore, bleaching was also observed under temperature stress in the dinoflagellate-bearing disk-shaped foraminifer *Marginopora vertebralis,* which showed increased mortality at 34°C and reduced photosynthesis at 31-32°C after one week of exposure (Uthicke et al. 2012) (Fig. 3). Bleaching has also been observed in the disk-shaped *Sorites* population from Florida and Belize (Richardson 2006; Richardson 2009), where local conditions suggested that fresh water runoff, hurricane events and high irradiance impact these populations. It has been suggested that bleaching susceptibility could depend on the collection depth (van Dam et al. 2012a), as species collected from slightly deeper habitats showed greater risk of bleaching (Schmidt et al. 2011; van Dam et al. 2012a; Schmidt et al. 2014). Marine invertebrates living in the inter-tidal zone have been shown to be more resistant to short-term temperature stress due to hosting a higher stability form of anti-oxidant enzymes than the invertebrates living in the sub-tidal zone (Regoli et al. 1997).

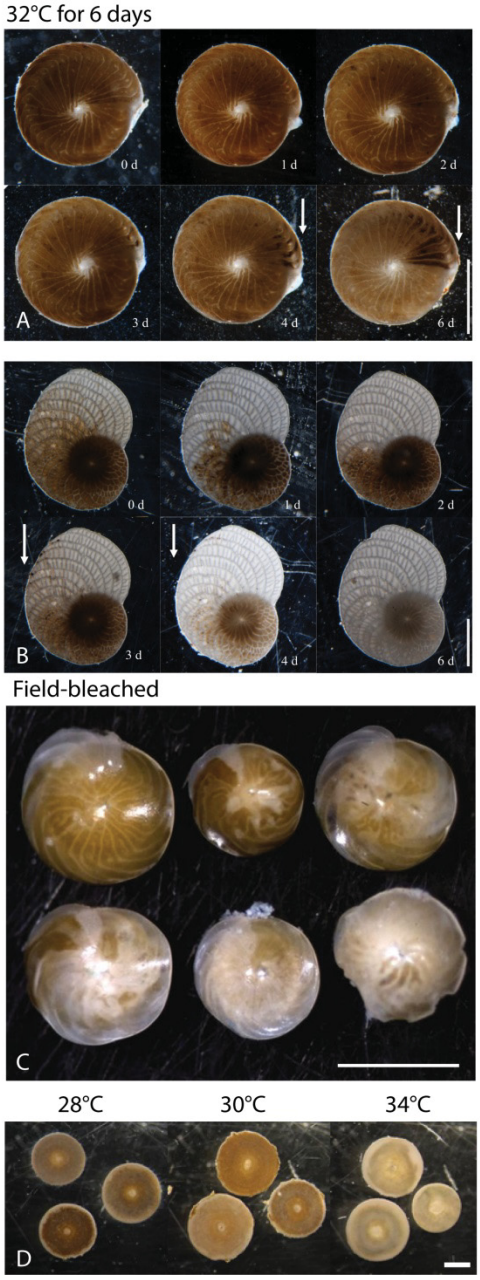


**Figure 3. Examples of bleaching in larger benthic foraminifera: A *Amphistegina radiata* and B *Heterostegina depressa* exposed to 32°C for 6 days from Whitsundays, Australia, symbiont loss is highlighted by arrows, see Schmidt et al. (2011), scale bar: 2 mm, C Examples of field-bleached *Amphistegina*, from Tennessee Reef, Florida Keys, USA, from summer 1999, images provided by P. Hallock, scale bar: 1mm, D Bleaching in *Marginopora vertebralis* from the Whitsundays, Australia (Shaw Island) after 7 days of exposure to treatment temperatures, images provided by S. Uthicke, see Uthicke et al. (2012), scale bar: 2 mm.**

## 1.4. Combined effects of global change

Since the industrial revolution, the world's oceans have been a sink for anthropogenic carbon dioxide. Under common carbon emission scenarios, this process is predicted to lower the oceanic pH between 0.3-0.5 units by 2100 (Caldeira and Wickett 2005; IPCC 2013). As a consequence, the saturation states (Ω) for calcium carbonate minerals (Orr et al. 2005; Doney et al. 2009) is lowered, reducing the carbonate ion concentration [$CO_2^{-3}$], which is essential for marine calcification (Feely et al. 2004; Kleypas and Langdon 2006). In addition, oceans are expected to warm at an increasingly rapid rate (IPCC 2013). Thus, ocean warming and ocean acidification (OA) are likely to act in combination impacting the calcification potential of many coral reefs (De'ath et al. 2009). It is not known whether the two stressors will combine, enhance or cancel the individual effects. Several studies suggested species-specific responses to combined effect of warming and acidification (Martin and Gattuso 2009; Koch et al. 2013). Recent meta-analysis combining those results showed the vulnerability of many species to a combination of stressors reducing key physiologic parameters such as growth, survival and calcification (Kroeker et al. 2013). The study by Kroeker et al. (2013) also showed that enhanced sensitivity of early life history stages is not universal among taxa. On the ecosystem level OA and warming are in combination lowering coral reef resilience (Anthony et al. 2011). The loss of ecological resilience occurs because coral regrowth is slow and disturbance with macro-algae increase in duration and frequency, so that coral-reef ecosystems are expected to shift from coral-dominated to algae-dominated reefs as a result of combined OA and warming (Hoegh-Guldberg et al. 2007; Carilli et al. 2009).

If ecosystem shifts from coral-reefs to algae-dominated reefs occur, we are expecting a change in light regimes and substrate, which will impact foraminifera associated with this habitat. Thus far, few studies with a focus on important carbonate sediment producers, such as LBF, have been conducted (Sinutok et al. 2011; Sinutok et al. 2014). The first study showed comprised photosynthetic health in the controls and needed to be repeated. The latter study showed combined effects of OA and warming reduced calcification and photosynthesis in *M. vertebralis* compared to the controls (Sinutok et al. 2014). Studies on the isolated effects of OA alone already showed mixed responses in LBF, which are best characterized as species-specific and dose dependent. LBF either show no reaction in manipulative

experiments (Vogel and Uthicke 2012; McIntyre-Wressnig et al. 2013), or reduced calcification (Kuroyanagi et al. 2009; Haynert et al. 2011; Reymond et al. 2013). Enhanced calcification in LBF was found until 770 µatm, possibly stimulated by carbon dioxide fertilization of the symbiont-population, but negative effects on calcification were observed >970 µatm (Fujita et al. 2011). This shows that the investigation of combined stressors on several different species of LBF is needed, especially with regard to the role different symbiont-types play in the stress response.

## 1.5. Culturing of symbiont-bearing foraminifera

Several laboratories have successfully cultured LBF to date, demonstrating that LBF are suitable organisms for answering ecological questions, which require experiments under controlled environmental conditions (Fujita et al. 2000; Talge and Hallock 2003; Fujita and Fujimura 2008; Nobes et al. 2008; Fujita et al. 2011; Hikami et al. 2011; Reymond et al. 2011; Schmidt et al. 2011; Uthicke et al. 2012; van Dam et al. 2012b; van Dam et al. 2012a; Reymond et al. 2013; Fujita et al. 2014; Schmidt et al. 2014). To be kept alive in culture LBF need light for their symbionts (Hallock 1981; Nobes et al. 2008), near constant salinity, nutrients in the form of nitrate or phosphate, or food given as living or dead microalgae (Lee et al. 1991) and temperatures not exceeding the summer maxima by 1-2°C of their natural habitat (Schmidt et al. 2011).

Röttger (1972a) started in the early 70's to culture LBF and described their life-cycle, chamber formation and feeding behavior (Röttger 1972c,a,b; Röttger and Berger 1972; Röttger 1973,1976). His work gave particular insights in biological functioning of the canal system in the species *Heterostegina depressa*, which is important for the motility, growth, reproduction and excretion (Röttger et al. 1984). Lee et al. (1979) reported the first successful isolation of the “naked” symbionts from LBF and grew them into culture. Diatoms have reduced their silica frustule inside their host, but re-grow it after death of their host when cultured in sterile media (Lee 1980; Lee et al. 1980). Experiments on the nutritional requirements of several LBF showed that those are species-specific and that they can take up nitrate and phosphate from the culturing seawater (Lee et al. 1991). Culturing experiments on *Amphistegina* showed that they develop a different test thickness when cultured under different water motions (Hallock et al. 1986). Moreover, LBF have been cultured to determine their response to different natural light levels, to test their photosynthetic and growth responses grown under daily fluctuating conditions (Nobes et al. 2008). Highest growth and photosynthetic rates ($F_v$:$F_m$) where found in all taxa in tanks when 90% of the incoming natural light was blocked by shade-cloth, with high light peaks of 60µmol photons $m^2$ $s^{-1}$ (Nobes et al. 2008). If LBF are cultured under light conditions similar to average light levels recorded by loggers in their shallow habitat (e.g. 200-300 µmol photons $m^2$ $s^{-1}$) they show reduced photosynthetic responses (reduced $F_v$:$F_m$), possibly because of photo-oxidative

stress (Sinutok et al. 2011). High irradiances exert stress on the photosymbiosis in LBF (Nobes et al. 2008), and based on their photosynthetic response to light, LBF can be categorized as being light-sensitive or light-tolerant (Ziegler and Uthicke 2011). As LBF are motile organisms, they can, unlike corals, shade themselves from light in their natural habitat during midday, similar to the cover behavior observed in many sea urchins (Adams 2001). With regard to adaptations for global change, mass culturing of the species *Baculogypsina sphaerulata* has been carried out and has successfully shown that it is a possible way to produce large amount of biogenic carbonate “artificially” which can be used for additional coastal stabilization (Hosono et al. 2014).

Several studies used flow-through aquaria systems for culturing LBF, which constantly provide new input of fresh seawater and/or nutrients (Reymond et al. 2011; Uthicke et al. 2012; van Dam et al. 2012a; Vogel and Uthicke 2012; Schmidt et al. 2014). This culturing method has been shown to be well suited for culturing over several weeks to month, especially because rapid water movement are also observed in the habitat of LBF (Williams and Carpenter 1998; Cornelisen and Thomas 2009) and high growth rate were reported in several species (Reymond et al. 2013; Schmidt et al. 2014). Earlier studies also reported positive influence of water motion on growth and calcification of LBF (ter Kuile and Erez 1984; Hallock et al. 1986). When large LBF are cultured in static-conditions, the lack of water movement around the shell could create boundary layer conditions, which can be thinned when water movement is applied. Increasing water exchange with the surrounding environment and can lead to changes in the pH induced by photosynthesis and calcification (Glas et al. 2012a). Thus, flow-through conditions for culturing, in contained in houses (Schmidt et al. 2014) or in free floating culture cages (Fujita et al. 2011), can be of advantage, especially with regard to testing effects of OA. For smaller LBF and juveniles the well-plate approach has been successfully used in studies monitoring the development of shells in asexual offspring (Chapter 2), for conducting direct measurements of photosynthesis using a Pulse Amplitude Fluorometer (Schmidt et al. 2011) or exposure tests to toxic metals (Prazeres et al. 2011) or herbicides (van Dam et al. 2012a), which provide a clear advantage during handling, reducing the loss of specimens.

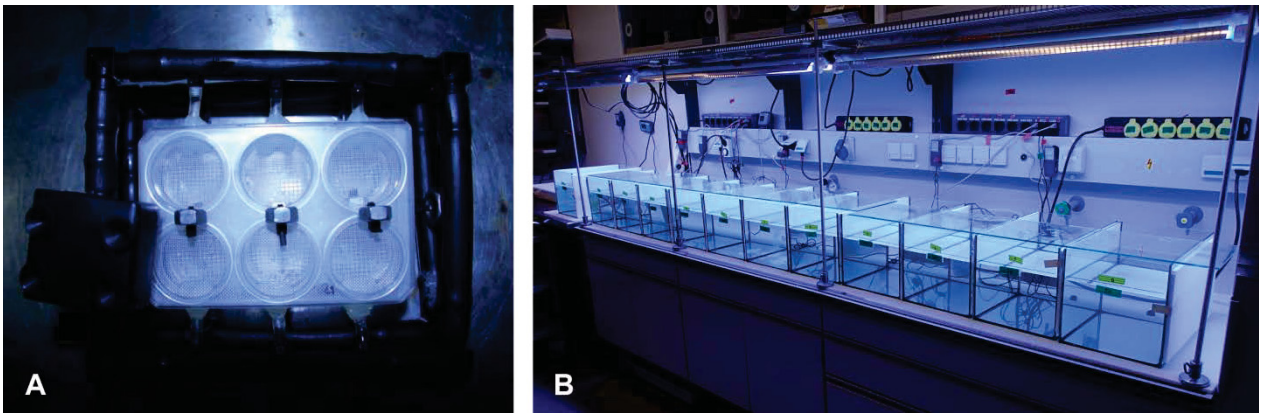


**Figure 4. Culturing setups for larger benthic foraminifera, A) Flow-through plate system used in Chapter 1 for culturing *Heterostegina depressa* and *Marginopora vertebralis*, made out of two standard 6-well plates, connected by plastic tubing to a small aquaria pump, *H. depressa* was contained in the lower level and *M. vertebralis* in the upper level for their optimal exposure to light, flow-through plates constructed to be submerged inside aquaria, B Aquaria setup to manipulate temperatures in each aquaria seperatly, which was used in Chapter 3 for temperature stress experiments (20-36°C).**

## 1.6. Aims and interdisciplinary research context

The overall goal of the thesis was to determine the physiological response of several species of LBF to individual and combined effects of global change stress. This was achieved by measuring the organisms' physiological responses by a variety of parameters, such as photosynthesis, respiration, chlorophyll a content, survivorship and growth. Understanding the effects of individual and combined stressors on marine ecosystems are increasingly important under global change scenarios, because stressors such as OA and warming are likely to occur simultaneously (Caldeira and Wickett 2005; IPCC 2013). We need to know whether theses stressors act additive (equal the sum of the individual effects), synergistic (larger than the sum of the individual effects) or antagonistic (erasing the individual effects) on marine species. We chose LBF because they are important calcium carbonate producers in the oceans and need further study. We wanted to test if differences exist between bleaching thresholds in different species and if they are lowered under a combination of stressors. Thus, we conducted a study on two LBF, hosting different photo-symbiotic dinoflagellates (*Marginopora vertebralis*) and diatoms (*Heterostegina depressa*). In addition to species from the Indo-pacific region LBF from the eastern Mediterranean Sea were chosen (Chapter 2) to test thresholds to bleaching on a larger geographic area. The eastern Mediterranean Sea is predicted to be a miniature model of ocean warming in the near future (Lejeusne et al. 2010; Shaltout and Omstedt 2014). In particular our attention was drawn to this area because an extreme thermally tolerant species was described by Arieli et al. (2011). They showed that the foraminifer *Pararotalia calcariformata*, can survive temperatures of 36°C, occurring in a heat plume originating from a power plant and that it likely contains endosymbionts (Arieli et al. 2011). This is remarkable, as all other eukaryote-eukaryote endosymbiosis including the most heat-tolerant corals are shown to bleach at 36°C (Coles 1988; Coles and Riegl 2013). We wanted to test whether the heat tolerance observed in situ is limited to the heat plume or also occurs in an unaffected population originating from a National Park. We wanted to test if the bleaching threshold of *P. calcariformata* is really higher than that of another abundant species *Amphistegina lobifera*, which has been shown to bleach starting at 31°C, and was not found in the heat plume (Talge and Hallock 2003; Schmidt et al. 2011). Therefore, we conducted several experiments, exposing a summer and winter

population of both species in the range from 20-36°C for up to three weeks and conducted an extreme heat-test experiment ranging from temperatures of 20-42°C on *Pararotalia*. We measured the species response by determining survival, growth and photosynthetic efficiency using Pulse Amplitude Modulated Fluorometry (PAM). We hypothesize that *P. calcariformata* is an invader species in the Mediterranean, as it was only recently described in the Levant (Reinhardt et al. 1994) and Turkish coast (Meriç et al. 2013). Hence, the aims were to characterize *P. calcariformata* combining morphological and molecular tools and describe its general ecology and reproduction cycle. We aimed to evaluate the species current and future distribution by 2100 using a species distribution model, previously used on a the genus Amphistegina in the Mediterranean (Langer et al. 2012; Weinmann et al. 2013). Furthermore, we wanted to test the temperature effect on the shell development of asexual juveniles to find out if *P. calcariformata* is restricted to a specific thermal tolerance window in their habitat, or if it is able to tolerate cold (20°C) or extremely warm (35°C) conditions in early life stages.

## 1.7. Working hypotheses

**Working hypotheses Chapter 2: Combined effects of warming and ocean acidification on coral reef foraminifera *Marginopora vertebralis* and *Heterostegina depressa.***

**A.) There is a difference between the individual and the combined effects of ocean acidification and warming on the physiology of *Heterostegina depressa* and *Marginopora vertebralis***

We tested this hypothesis by experimentally exposing *H. depressa* and *M. vertebralis* to elevated temperatures and elevated carbon dioxide concentrations in a multi-factorial experiment. This experiment consisted of four treatments, involving a combination of two $pCO_2$levels (equivalent to pH 7.9 and 8.1) and two temperature levels (28 and 31°C). In particular, we aimed to gain a better understanding of the effects of the combined stressors on both the photo-symbionts (photosynthesis, oxygen production, and chlorophyll a content) and the holobiont (survivorship, respiration, and growth).

**B.) The combined effects are additive, synergistic or antagonistic**

When combined effects were significant based on general linear models, we calculated the observed inhibition compared to the control treatment, based on this we described if the effect is additive (the sum of the individual effects), synergistic (larger than the sum of the individual effects) or antagonistic.

**C.) The response to the combined effects is species-specific**

We chose particular species with different symbiont types (diatoms and dinoflagellates) to evaluate the difference between species with regard to differentiated response to the interaction of stressors.

**Working hypotheses Chapter 3: Recent invasion of the symbiont-bearing foraminifera *Pararotalia* into the Eastern Mediterranean facilitated by the ongoing warming trend**

**A.) Based on its current distribution, the recently discovered foraminifer *P. calcariformata* in the eastern Mediterranean Sea is an invader species**

We characterized its morphology and current biogeography by identifying its current distribution in the eastern Mediterranean Sea. To investigate whether this species is a likely invader we analysed the relatedness of Mediterranean and Indo-Pacific population using phylogenetic inference. Using a compilation of all occurrence records of the species in the Mediterranean, we model its likely current distribution.

**B.) The foraminifer *P. calcariformata* contains permanent diatom endosymbionts**

We also identified its endosymbiotic microalgae by molecular and standard algae culturing and measured its photosynthetic activity under controlled environmental condition.

**C.) The symbionts in *P. calcariformata* are photosynthetically active over several month in culture**

We cultured this species over five month and monitored its photosynthetic activity using Pulse Amplitude modulated Fluorometry, as well measured its response to rapidly increasing light conditions to evaluate its optimal light properties for culturing.

**D.) The foraminifer *P. calcariformata* will spread to currently colder regions in the Mediterranean based on global warming**

Based on the current occurrence records and the predictions that the distribution of species is likely impacted by the minimal temperature, turbidity and radiation we model its future spread under a global change scenario.

**E.) *Pararotalia calcariformata* has a narrow reproductive window for asexual offspring development and is currently restricted by minimum temperatures to spread westwards**

We hypothesized that temperature is the main factor for the establishment of new populations, as it is seen worldwide in most shallow marine fauna (Belanger et al. 2012). To test if there are temperature differences between the developmental rates in *Pararotalia* juveniles, we exposed them to three different temperatures (20°C, 28°C and 35°C). We measured growth rates to find out which temperatures promote shell development and growth in this species.

**Working hypotheses Chapter 4: Extreme heat tolerance of a foraminifera–diatom photo-symbiosis**

**A.) *Pararotalia calcariformata* shows a unique thermal tolerance, with symbionts performing photosynthesis at temperatures up to 36°C, which could be an explanation why it occurs in the heat-plume**

To test these hypotheses, we collected *P. calcariformata* and *A. lobifera* from a natural unpolluted habitat. The genus Amphistegina was chosen for comparison, as it populates the same habitat, and also because it has been shown to bleach at temperatures of 32°C (Talge and Hallock 2003; Schmidt et al. 2011). *Pararotalia calcariformata* was observed in the heat plume of a power plant to withstand temperature of 36°C (Arieli et al. (2011). So we conducted an experiment using the summer population exposing both species to temperatures from 24°C-35°C and measured growth and photosynthetic activity for two weeks.

**A) The winter population is more sensitive than the summer population to elevated temperatures**

We tested the above hypothesis by repeating the above experiment for three weeks with populations collected after the winter month (20°C-36°C) to see if their physiological response is changing, and the photo-symbiosis would show different signs of stress, when species are not pre-adapted naturally to summer temperatures.

**B) The foraminifer *P. calcariformata* has the same general thermal limit of bleaching of 1-3°C above observed current summer maxima, compared to corals and other eukaryotic photosymbioses**

To test this we re-sampled the summer population of *P. calcariformata* in the following year. For defining the species general thermal limit of the photosynthetic activity we conducted an experiment exposing it to four different temperatures in the range of 20-42°C for the duration of three weeks. We measured again the photosynthetic activity and growth rates. We then compared the results to other eukaryote-eukaryote symbiosis.

## 1.8. Outline of the thesis

Publication 1:**Schmidt, C.,** Kucera, M., Uthicke, S.

**Combined effects of warming and ocean acidification on coral reef Foraminifera *Marginopora vertebralis* and *Heterostegina depressa.*** The article has been published in the Journal Coral Reefs (2014) 33(3):805-818.

Contributions: The project on interactive effects of ocean acidification and warming on LBF was initiated by S. Uthicke and the AIMS water quality team. Planning of the particular project on foraminifera was done by S. Uthicke and C. Schmidt. The experiment was carried out by S. Uthicke, C. Schmidt , M. Liddy, A. Negri, N. Webber, K. Fabricius, S. Noonan, as a collaborative project. The physiological data on LBF was collected by C. Schmidt. Data analysis was done by C. Schmidt with help of S. Uthicke. Writing of the manuscript was done by C. Schmidt with improvements by M. Kucera and S. Uthicke.

Publication 2: **Schmidt, C,** Morard, R, Almogi-Labin, A, Weinmann, A, Titelboim, D, Abramovich, S, Kucera, M

**Recent invasion of the symbiont-bearing Foraminifera *Pararotalia* into the Eastern Mediterranean facilitated by the ongoing warming trend**

Contributions: The hypotheses tested in this paper were developed by M. Kucera and C. Schmidt. Molecular data collection and analysis was conducted by R. Morard. Data collection on the biology of *Pararotalia* (photosynthetic measurements, symbiont isolation, juvenile development, occurrence records) was done by C. Schmidt. A. Weinmann conducted species distribution modelling based on occurrence records. D. Titelboim contributed to the description of the taxonomy. Writing of the manuscript was done by C. Schmidt and M. Kucera with improvements by A. Almogi-Labin and S. Abramovich.

Publication 3: **Schmidt, C.,** Brandt, J., Barak, H., Abramovich, S., and Kucera, M.

**Extreme heat tolerance of a foraminifera–diatom photo-symbiosis**

Contributions: The hypotheses and experimental design were developed by C. Schmidt and M. Kucera. Experimental data was collected by C. Schmidt and J. Brandt. B. Herut and S. Abramovich contributed to field background temperature data collection. Data analysis was done by C. Schmidt, and M. Kucera. C. Schmidt wrote the paper, with improvements by M. Kucera.

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# Chapter 2

# 2. Publication I: Combined effects of warming and ocean acidification on coral reef foraminifera *Marginopora vertebralis* and *Heterostegina depressa Coral Reefs*

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REPORT

# Combined effects of warming and ocean acidification on coral reef Foraminifera *Marginopora vertebralis* and *Heterostegina depressa*

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**Abstract** Warming and changes in ocean carbonate chemistry alter marine coastal ecosystems at an accelerating pace. The interaction between these stressors has been the subject of recent studies on reef organisms such as corals, bryozoa, molluscs, and crustose coralline algae. Here we investigated the combined effects of elevated sea surface temperatures and $pCO_2$ on two species of photosymbiont-bearing coral reef Foraminifera: *Heterostegina depressa* (hosting diatoms) and *Marginopora vertebralis* (hosting dinoflagellates). The effects of single and combined stressors were studied by monitoring survivorship, growth, and physiological parameters, such as respiration, photochemistry (pulse amplitude modulation fluorometry and oxygen production), and chl *a* content. Specimens were exposed in flow-through aquaria for up to seven weeks to combinations of two $pCO_2$ ($\sim$790 and $\sim$490 µatm) and two temperature (28 and 31 °C) regimes. Elevated temperature had negative effects on the physiology of both species. Elevated $pCO_2$ had negative effects on growth and apparent photosynthetic rate in *H.depressa* but a positive effect on effective quantum yield. With increasing $pCO_2$, chl *a* content decreased in *H. depressa* and increased in *M. vertebralis*. The strongest stress responses were observed when the two stressors acted in combination. An interaction term was statistically significant in half of the measured parameters. Further exploration revealed that 75 % of these cases showed a synergistic (= larger than additive) interaction between the two stressors. These results indicate that negative physiological effects on photosymbiont-bearing coral reef Foraminifera are likely to be stronger under simultaneous acidification and temperature rise than what would be expected from the effect of each of the stressors individually.

**Keywords** Climate change · Ocean acidification · Benthic Foraminifera · Diatoms · Dinoflagellates · Symbiosis

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## Introduction

Coral reef ecosystems react sensitively to rapid climatic events and changes in ocean carbonate chemistry (Hoegh-Guldberg et al. 2007; Wernberg et al. 2013). It is increasingly acknowledged that an understanding of the effect of these stressors on marine organisms requires experiments investigating the combined effects of multiple stressors. In theory, when the stressors individually have a negative effect, then their combined effect can be either additive (total effect $C = A + B$), antagonistic ($C < A + B$), or synergistic ($C > A + B$) (Crain et al. 2008). In the context of global change, it is especially important to understand whether synergistic effects are likely to occur under the combination of stressors. Interactive effects of warming and rising $pCO_2$ have been observed in marine organisms, such as corals, bryozoa, molluscs, and crustose coralline algae (Reynaud et al. 2003; Anthony et al. 2008;

Rodolfo-Metalpa et al. 2010, 2011). A meta-analysis of a decade of climate change experiments on marine organisms indicated that environmental stressors can have significant combined effects, often not detectable in single-stressor studies (Wernberg et al. 2012). The negative effects of raised $pCO_2$ are amplified by simultaneously elevated temperatures in marine crustose coralline algae and macroalgae, but indicate that the response to the combined treatment is species specific (Martin and Gattuso 2009; Koch et al. 2013). In contrast, McCulloch et al. (2012) showed that increasing temperature may counteract the negative effects of acidification on calcification in corals, by facilitating upregulation of pH at the site of calcification. Collectively, these studies illustrate that coral reef organisms exhibit significant interactive effects in response to key global change stressors, but the strength and direction of response differ among taxa.

Pörtner (2002, 2008) argued that protists and especially prokaryotes might be less vulnerable to $pCO_2$ and temperature stress than more complex macro-organisms because the latter are more specialised on a molecular level. An ecologically significant group of protists on coral reefs are benthic Foraminifera. Foraminifera are major ecosystem engineers in coral reefs because they contribute significantly to the carbonate sediment production (Langer et al. 1997), providing substrata for other coral reef organisms. Larger Foraminifera host photosymbionts, which facilitate growth to cell sizes 10–100 times larger than their asymbiotic relatives (Hallock 1985; Lee and Hallock 1987; Lee 1995). The endosymbiosis is sensitive to thermal and light stress, leading to bleaching analogous to that in corals (Hallock et al. 1992; Hallock and Talge 1993). The effect of thermal stress in Foraminifera has been documented in laboratory studies (Talge and Hallock 2003; Schmidt et al. 2011; Uthicke et al. 2011). In the field, thermal pollution was documented to effect foraminiferal species composition (Arieli et al. 2011).

The effect of changes in ocean carbonate chemistry on Foraminifera due to raised $pCO_2$ levels in the atmosphere is not yet fully understood. Experimental manipulations of various species of Foraminifera using $pCO_2$ levels of up to 2,000 µatm have shown no evidence for reduced survivorship (McIntyre-Wressnig et al. 2013), nor any effects on photobiology and calcification (Vogel and Uthicke 2012), supporting the hypothesis of Pörtner (2002, 2008). Other studies, however, have shown reduced calcification of coral reef Foraminifera at elevated $pCO_2$ levels (Kuroyanagi et al. 2009; Haynert et al. 2011; Reymond et al. 2013). Fujita et al. (2011) reported that calcification in two tropical Foraminifera hosting diatoms and exhibiting a hyaline shell increased with elevated $pCO_2$ up to 770 µatm and decreased at $pCO_2$ levels up to 970 µatm. One species with a porcelaneous shell decreased in size with increasing $pCO_2$. Species-specific responses have been confirmed in a study by Hikami et al. (2011). Studies of Foraminifera from natural $CO_2$ seeps with locally decreased pH reported increasing incidences of altered shell structure, decreasing population densities, declining diversity in calcifying Foraminifera, and increasing proportion of Foraminifera with agglutinated shells towards low pH/high $pCO_2$ (Dias et al. 2010; Fabricius et al. 2011; Uthicke and Fabricius 2012; Uthicke et al. 2013). Test dissolution has been reported under elevated $pCO_2$ (Sinutok et al. 2011).

Interactive effects of elevated $pCO_2$ and eutrophication have been shown to impact growth in *Marginopora rossi* (Reymond et al. 2013). Elevated temperatures and eutrophication can also have interactive negative effects on growth and survivorship in *Marginopora vertebralis* (Uthicke et al. 2011). Negative effects of elevated temperatures are more severe in the presence of low concentrations of the herbicide Diuron (van Dam et al. 2012).

The main aim of this study was to investigate the individual effects of $pCO_2$ and temperature and their combined effects on coral reef Foraminifera. In particular, we aimed to gain a better understanding of parameters targeting the foraminiferal photosymbionts (photosynthesis, oxygen production, and chl *a* content) and the holobiont (survivorship, respiration, and growth) in response to a combination of stressors. The multi-factorial experiment consisted of two $pCO_2$ levels (pH 7.9 and 8.1) and two temperature levels (28 and 31 °C). The elevated treatments (31 °C and pH 7.9) simulate levels that are predicted to occur by the end of the century in Australian coastal waters (Lough and Hobday 2011; Redondo-Rodriguez et al. 2012). Since investigations of multiple stressors on other marine organisms showed significant differences among taxa, the experiment included two species of Foraminifera. *Heterostegina depressa* and *M. vertebralis* represent two phylogenetically distinct clades that appear to have diverged >500 million years ago, evolved calcification independently of each other (Pawlowski et al. 2003) and host different types of photosymbionts (Lee 2006).

## Materials and methods

### Species selection and sample collection

Two species of Foraminifera were investigated: *M. vertebralis* and *H. depressa*. *M. vertebralis* represents the family Soritidae, which produces imperforate, porcelaneous tests made of high-Mg calcite (Blackmon and Todd 1959). This species is abundant in shallow reef settings of the Indo-Pacific Ocean (Langer and Lipps 2003). It harbours dinoflagellate symbionts of the genus *Symbiodinium*

(Pochon et al. 2007; Momigliano and Uthicke 2013). *H. depressa* is a representative of the family Nummulitidae, which produces multi-layered, perforate low Mg-calcite tests and harbours endosymbiotic diatoms (Lee et al. 1980; Leutenegger 1984). This species occurs in highest abundance below 10-m water depth and is less abundant in highly energetic shallow habitats (Hohenegger et al. 1999; Renema 2006; Nobes et al. 2008). Specimens were collected from Orpheus Island in the central Great Barrier Reef in September 2011. *H. depressa* was collected at a depth of 8–12 m from coral rubble (Cattle Bay, 18°34′08″S 146°28′55″E) and *M. vertebralis* at a depth of 0–1 m (below Lowest Astronomical Tide) from turf algae-covered rocks (Hazard Bay, 18°38′58″S 146°29′11″E). Both species were acclimated to laboratory conditions in tanks with moderate flow-through conditions (same as used in experimental setup) under low-light conditions (10 µmol photons $m^{-2}$ $s^{-1}$) for a period of 3 weeks.

## Experimental design and carbonate system parameters

12 flow-through aquaria (working volume 17.5 L) were installed in a constant temperature room, and the experiment was carried out over a period of 53 d. The duration of experimental exposure was adapted to the species' biological response in order to perform final respiration and production measurements on all treatments. The exposure time of 53 d for *M. vertebralis* and 35 d for *H. depressa* was deemed adequate to observe the effects of long-term stress. For each temperature (28 and 31 °C) and $pCO_2$ level (~790 µatm, $pH_{NIST}$ 7.9 and ~490 µatm, $pH_{NIST}$ 8.1), three replicate tanks were used; replicate tanks were randomly allocated to treatments. Seawater was pumped from the ocean into the laboratory, filtered to 5 µm, and stored in four header tanks where it was modified to the desired experimental conditions. Temperature was manipulated through a computer-controlled data logger (CR 1000, Campbell Scientific, Australia). Titanium heating rods in the four header tanks heated the incoming seawater to the set temperatures, which were monitored by the data logger in each header tank and in one aquarium per treatment. The seawater was then pumped into the aquaria at a flow rate of 450–500 mL $min^{-1}$ (determined by flow indicators, RS Components, Ltd., UK). Manual temperature and pH measurements were performed once to twice per day (Table 1), using a Eutech, USA, probe and Oakton, USA, console. For the increased $pCO_2$ treatment, water chemistry was manipulated by bubbling analytical $CO_2$ into the header tanks. The water chemistry was controlled by a computer aquarium system (Aquamedic, Germany), as described in Uthicke et al. (2011) and Vogel and Uthicke (2012). Water samples for total alkalinity ($A_T$) and dissolved inorganic carbon (DIC) determinations were taken weekly and analysed by AIMS Laboratory Services (Vindta 3C). The program CO2 SYSCALC.EXE (Lewis and Wallace 1997) was used to calculate carbonate system parameters from $A_T$, DIC, salinity, and temperature values (Table 1).

## Experimental approach mimicking 'natural' conditions

Specimens were kept inside custom made flow-through housings in each aquarium to achieve higher flow conditions more closely mimicking their habitat than in previous experiments (Schmidt et al. 2011; Uthicke et al. 2011; Vogel and Uthicke 2012). Flow-through housings contained two levels made from two standard 6-well cell-culturing plates with flow-through lids (Electronic Supplementary Material, ESM Fig. S1). Twenty-four specimens (four specimens per well) of *H. depressa* were put in the lower level and the same number of *M. vertebralis* in the top level. Foraminifera were contained in the housings by placing a plankton mesh (Ø 0.5 mm: *H. depressa*, Ø 1 mm: *M. vertebralis*) and additional shading cloth between plate and lid, held tight by rubber bands (ESM Fig. S2). For the construction of the housings, six larger circles (Ø 3.5 cm) were cut into the lids as water outlets and six smaller circles (Ø 0.3 cm) on each side as water inlets. Black plastic tubing was used to space the plates 1 cm apart and to connect them horizontally and vertically. At the top end, a small aquarium pump delivered a constant flow of water from the aquarium to the inside of the housings. Flow into each well was visible because small flow indicators (1-cm-long red ribbons glued at one end inside each inlet of the housings) were constantly held in place by the flowing water. Flow rates into the individual wells were recorded before and after the experiment, ranging between 180 and 220 mL $min^{-1}$. Velocity of the water flow varied from 4.2–5.2 × $10^{-1}$ m $s^{-1}$ at the inlet (Ø 0.3 cm) to 3.1–3.8 × $10^{-3}$ m $s^{-1}$ at the outlet (Ø 3.5 cm). The flow rates between inlet and outlet are in the same range as those measured in situ over dead coral rubble (Williams and Carpenter 1998) and sea grass (Cornelisen and Thomas 2009). Both species kept in the flow-through housings appeared to thrive as indicated by healthy colouration, minimal shell breakage, and the development of firm attachment to the walls of the housings by the pseudopodial network. *H. depressa* was attached to the walls of the housings, whereas *M. vertebralis* was attached to the flat bottom of the housings, mimicking its firm attachment and its natural position on sea grass leaves. The flow-through system had the disadvantage that a small number of specimens were lost during the experiment (mean specimen loss per treatment: *H. depressa*: 5–13 %, SD = 0–10 %; *M. vertebralis* 0–6 %, SD = 0–7 %).

**Table 1** Carbonate system parameters over the course of the experiment

| Treatment | Measured parameters | | | | Calculated parameters | | |
|---|---|---|---|---|---|---|---|
| | $pH_{NIST}$ (SD) | Temperature (°C) (SD) | $A_T$ (μmol $kg^{-1}$ SW) | DIC (μmol $kg^{-1}$ SW) | $pCO_2$ (μatm) (SD) | $\Omega_{Ca}$ (SD) | $\Omega_{Ar}$ (SD) |
| 28, 8.1 control | 8.15 (0.05) | 28.1 (0.2) | 2332 (24) | 2031 (10) | 479 (38) | 5.1 (0.3) | 3.4 (0.2) |
| 31, 8.1 elevated *T* | 8.14 (0.05) | 30.8 (0.3) | 2338 (20) | 2025 (6) | 499 (32) | 5.4 (0.3) | 3.6 (0.2) |
| 28, 7.9 elevated $pCO_2$ | 7.98 (0.05) | 27.9 (0.3) | 2335 (22) | 2134 (19) | 738 (65) | 3.8 (0.2) | 2.5 (0.1) |
| 31, 7.9 elevated temp & $pCO_2$ | 7.96 (0.03) | 30.8 (0.4) | 2337 (22) | 2142 (16) | 835 (85) | 3.8 (0.3) | 2.6 (0.2) |

pH and temperature values were derived from individual daily measurements ($N = 49$), including light and dark cycle, whereas water chemistry parameters $A_T$ and DIC were measured from two sets of experimental samples taken over the course of the experiment to calculate $pCO_2$, $\Omega_{Ca}$ and $\Omega_{Ar}$

### Experimental light levels

Flow-through housings were made containing two levels so that one species could be kept in the top level exposed to higher light levels than the species in the lower level. Light levels were chosen for each species separately because of their known distributions and different light saturation points determined by pulse amplitude modulation (PAM) fluorometry (Hohenegger 2004; Ziegler and Uthicke 2011; Vogel and Uthicke 2012). PAM fluorometry results for *M. vertebralis* indicated a maximum saturating irradiance ($E_k$) between 100 and 140 μmol photons $m^{-2}$ $s^{-1}$ and for *H. depressa* between 40 and 60 μmol photons $m^{-2}$ $s^{-1}$ (Ziegler and Uthicke 2011). The light levels used in the experiment were chosen to fall below the $E_k$ values by Ziegler and Uthicke (2011) and *P–I* curve $P_{max}$ values determined by Vogel and Uthicke (2012) and were selected to correspond to levels which have shown no changes in mortality rates or chlorophyll *a* concentrations in previous experimental manipulations of the studied species (Schmidt et al. 2011; Uthicke et al. 2011; Vogel and Uthicke 2012; Reymond et al. 2013). *H. depressa* was put in the lower level (10–17 μmol photons $m^{-2}$ $s^{-1}$) of the flow-through housings and *M. vertebralis* in the top level (38–45 μmol photons $m^{-2}$ $s^{-1}$) because the latter has a higher $E_k$ point, compared with the diatom-bearing species (Ziegler and Uthicke 2011). Overall, light levels inside the tanks (140–150 μmol photons $m^{-2}$ $s^{-1}$) were recorded at the beginning and the end of the experiment using a light quantum sensor (Apogee MQ-200, USA). Light was supplied by 50:50 actinic 420 nm/10 K trichromatic daylight fluorescent grow tubes (Catalina Compact, 12-h dark/12-h light cycle). Green shade cloth (light reduction by ~30 %) was used for *M. vertebralis,* and black shade cloth (light reduction by ~50 %) was used for *H. depressa*. Shade cloth and plankton mesh were exchanged every week to keep light levels constant over the experimental period.

### Survivorship and growth

To determine % survivorship, specimens were examined twice per week and recorded as living, when their cytoplasm exhibited colour, or dead, when the shells and cytoplasm were pale and no cytoplasmic activity was observed (Bernhard 2000). Growth of *H. depressa* and *M. vertebralis* was expressed as the increase in cross-sectional surface area per day over the experimental period. Procedures of high-resolution photography before and at the end of the experiment were the same as previously published (Uthicke and Altenrath 2010; Schmidt et al. 2011; Vogel and Uthicke 2012). Surface area ($mm^2$) of individual Foraminifera was measured and analysed as described in Schmidt et al. (2011). *H. depressa* growth rates have been based on tracking individuals within one well from the beginning to the end of the experiment. This was possible by following distinct shell features and overall size among the images through time. *M. vertebralis* growth rates had to be based on overall means of wells because the shells of this species do not possess characteristic differences that would allow the tracking of individual specimens. Wells where *M. vertebralis* were lost were excluded from the data set, as were wells where mechanical damage to the specimens occurred. Growth rates (% $d^{-1}$) were determined following the equation of ter Kuile and Erez (1984). Average initial surface area of analysed specimens did not deviate between treatments both with respect to the mean values and the variance (*H. depressa*: one-way ANOVA, $F_{1,143} = 0.29$, $p = 0.8346$; Levene's Test, $F_{3,142} = 1.22$, $p = 0.304$; *M. vertebralis*: one-way ANOVA, $F_{1,57} = 1.10$, $p = 0.3559$; Levene's Test, $F_{3,53} = 1.077$, $p = 0.366$).

### Photobiology, oxygen consumption, and chlorophyll *a* concentration

Photochemical performance of Photosystem II (PSII) was measured by obtaining the maximum quantum yield

(MQY, dark adapted yield, $F_v$:$F_m$) and the effective quantum yield (EQY, light adapted yield, ΦPSII) of individual *H. depressa* and *M. vertebralis* before and at the end of the experiment with an Imaging-PAM Fluorometer (WALZ, Unit IMAG-CM, Maxi Head, Germany; Schmidt et al. 2011; Uthicke et al. 2011; Vogel and Uthicke 2012). Foraminifera were transferred into 6-well plates containing the respective treatment water and dark adapted for 20 min prior to measuring MQY. Similar light conditions as in the aquaria, supplied by the LED unit of the Maxi head (*H. depressa*, 10–14 µmol photons $m^2$ $s^{-1}$, *M. vertebralis* 35–40 µmol photons $m^2$ $s^{-1}$), were used to measure EQY. Calculations of EQY (ΦPSII) and MQY ($F_v$:$F_m$) were conducted by the software Imaging Win (WALZ, Germany) after independent AOI (Areas of Interest) were put on the individual Foraminifera from which the measurements were read. For further information on the Imaging-PAM, details are described in Hill et al. (2004). Additionally, PAR-absorptivity (Abs) was measured as the ratio of reflectance of red light (650 nm, *R*) to the reflectance of non-absorbed near-infrared light (780 nm, NIR) from individual Foraminifera at the end of the experiment. This is based on the assumption that the change in photosynthetic pigments within the same species will alter the absorption of red light and change the ratio. For calculating the apparent photosynthetic rate (APR, Ps), we used the formula: Abs × EQY × light intensity (Cooper and Ulstrup 2009). Absorptance measurements are used to calculate APR and are influenced by differences in the calcareous shells of the species. Therefore, affecting absolute and relative differences and are thus only discussed in light of relative changes in response to the tested environmental parameter and not used to compare absolute values between the species.

Respiration and photosynthesis rates were determined in incubation chambers by measuring changes in oxygen concentration during a 15-min dark phase followed by a 15-min light phase, using a custom-build respirometer (Uthicke et al. 2011; Vogel and Uthicke 2012), before and after the experiment. Non-invasive oxygen sensor spots ('optodes', Ø 5 mm) were attached to closed glass vials (volume 6.6 mL) containing the Foraminifera. Fibre optic cables connected these to an OXY-4 mini transmitter (Presens, Germany). Prior to measurements, specimens were incubated in the dark for a minimum of 25 min to stabilise temperatures in the flow-through water bath. For photosynthesis measurements, Foraminifera were exposed to the same light conditions as used in the experiment (light sources: Catalina compact: 420 nm actinic/1,000 K). In each run, one out of four vials did not contain any species, to test for potential respiration not caused by the Foraminifera. Prior to the experiment, baseline respiration and production rates were measured in the ambient $pCO_2$ and temperature treatment. For each of these replicates, prior to and at the end of the experiment, 3 specimens of *M. vertebralis* and 6–8 specimens of *H. depressa* were pooled to obtain a sufficiently strong signal. For final measurements, five replicates were measured per aquarium for *M. vertebralis* and two for *H. depressa*. Respiration rates were normalised to wet weight (determined to 0.01 mg accuracy with balance, Mettler-Toledo), which is known to be highly correlated with dry weight in Foraminifera (Schmidt et al. 2011). Daily net production rates were calculated assuming that the respiration rates reflect a 12-h night cycle and production rates the 12-h day cycle. To determine how the observed changes in photosynthetic performance are linked with pigment content of the whole organism, the average concentration of chlorophyll *a* was determined at the end of the experiment for a subset (four specimens) per aquaria. Chlorophyll *a* was extracted and quantified following the protocol described in Schmidt et al. (2011).

## Data analysis

For statistical analyses, MQY ($F_v$:$F_m$), EQY (ΦPSII), growth, and survivorship were arc sine transformed because they represent proportions or percentages. Oxygen respiration and production were log $(x + 1)$ transformed and chl *a* pigment data were log transformed to meet the assumption of the ANOVA. Residual and normality plots on transformed data indicated that assumptions of equal group variance and normality were not violated. Changes in all parameters under the respective temperature and $pCO_2$ treatments were analysed using linear models, into which average temperature and pH for each individual aquarium obtained through manual measurements were inserted as factors including their interaction term. The analysis of MQY, EQY, and APR was based on average values per well, yielding each a total of 72 values (6 wells * 4 treatments * 3 replicate tanks). Analysis of respiration parameters was based on two replicate measurements per tank for *H. depressa* ($N = 24$) and four replicate measurements per tank for *M. vertebralis* ($N = 48$). Analysis of chl *a* content was based on four replicate measurements per tank (4 specimen * 4 treatments * 3 replicates, $N = 48$). Analysis of survivorship was based on values for each of the three replicate tanks ($N = 3$). Analysis of growth rates was based on averages per well for *H. depressa* ($N = 68$) and for *M. vertebralis* ($N = 57$).

All analyses were conducted in Jmp, Version 10 (SAS 2012). In cases where linear models indicated significant effects in both parameters, or a significant interaction between the stressors, we calculated the expected additive inhibition from each individual parameter according to a standard ecotoxicological model (Bliss 1939) to compare observed effects with expected effects. We determined

**Table 2** Linear model analysis of the effect of all physiological parameters based on values at the end of the experiment with *Heterostegina depressa* (after 35 days) and *Marginopora vertebralis* (after 53 days)

| Parameter | Factor | *Heterostegina depressa* | | | | | *Marginopora vertebralis* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Estimate | SE | *t* | *p* | $R^2$ | Estimate | SE | *t* | *p* | $R^2$ |
| MQY ($F_v$:$F_m$) | Intercept | 0.794 | 0.100 | 7.920 | **0.000** | 0.512 | 0.915 | 0.105 | 8.720 | **0.000** | 0.674 |
| | Temp | −0.005 | 0.001 | −7.280 | **0.000** | | −0.009 | 0.001 | −11.420 | **0.000** | |
| | $pCO_2$ | 0.022 | 0.012 | 1.870 | 0.066 | | 0.015 | 0.013 | 1.220 | 0.228 | |
| | Temp × $pCO_2$ | 0.021 | 0.008 | 2.560 | **0.013** | | 0.013 | 0.009 | 1.510 | 0.135 | |
| EQY (ΦPSII) | Intercept | 2.095 | 0.319 | 6.580 | **0.000** | 0.367 | 1.341 | 0.300 | 4.460 | **0.000** | 0.838 |
| | Temp | −0.025 | 0.002 | −10.530 | **0.000** | | −0.042 | 0.002 | −18.530 | **0.000** | |
| | $pCO_2$ | −0.089 | 0.038 | −2.340 | **0.022** | | 0.039 | 0.036 | 1.080 | 0.286 | |
| | Temp × $pCO_2$ | −0.077 | 0.027 | −2.900 | **0.005** | | −0.092 | 0.025 | −3.680 | **0.001** | |
| APR (Ps) | Intercept | 11.594 | 3.398 | 3.410 | **0.001** | 0.851 | 49.862 | 16.798 | 2.970 | **0.004** | 0.781 |
| | Temp | −0.489 | 0.026 | −19.100 | **0.000** | | −1.949 | 0.127 | −15.400 | **0.000** | |
| | $pCO_2$ | 0.931 | 0.404 | 2.300 | **0.025** | | 2.296 | 2.006 | 1.140 | 0.256 | |
| | Temp × $pCO_2$ | −0.014 | 0.283 | −0.050 | 0.961 | | −1.039 | 1.399 | −0.740 | 0.460 | |
| Respiration | Intercept | −0.001 | 0.024 | −0.050 | 0.963 | 0.304 | 0.000 | 0.043 | 0.010 | 0.992 | 0.608 |
| | Temp | 0.000 | 0.000 | 2.650 | **0.016** | | 0.002 | 0.000 | 5.960 | **0.000** | |
| | $pCO_2$ | −0.002 | 0.003 | −0.730 | 0.474 | | −0.009 | 0.005 | −1.760 | 0.086 | |
| | Temp × $pCO_2$ | 0.001 | 0.002 | 0.540 | 0.593 | | −0.012 | 0.004 | −3.440 | **0.001** | |
| Production | Intercept | 0.037 | 0.135 | 0.270 | 0.788 | 0.587 | 0.150 | 0.136 | 1.110 | 0.274 | 0.399 |
| | Temp | −0.005 | 0.001 | −4.820 | **0.000** | | −0.005 | 0.001 | −4.910 | **0.000** | |
| | $pCO_2$ | 0.016 | 0.016 | 1.000 | 0.329 | | 0.002 | 0.016 | 0.150 | 0.882 | |
| | Temp × $pCO_2$ | 0.005 | 0.011 | 0.470 | 0.644 | | 0.009 | 0.011 | 0.780 | 0.441 | |
| Net production | Intercept | 0.038 | 0.146 | 0.260 | 0.796 | 0.592 | 0.162 | 0.148 | 1.090 | 0.282 | 0.521 |
| | Temp | −0.005 | 0.001 | −4.860 | **0.000** | | −0.007 | 0.001 | −6.070 | **0.000** | |
| | $pCO_2$ | 0.018 | 0.017 | 1.040 | 0.313 | | 0.009 | 0.018 | 0.530 | 0.602 | |
| | Temp × $pCO_2$ | 0.004 | 0.012 | 0.350 | 0.730 | | 0.020 | 0.012 | 1.620 | 0.114 | |
| Chl α content | Intercept | 2.377 | 2.609 | 0.910 | 0.368 | 0.799 | −11.910 | 5.006 | −2.380 | **0.022** | 0.712 |
| | Temp | −0.233 | 0.020 | −11.880 | **0.000** | | −0.316 | 0.038 | −8.370 | **0.000** | |
| | $pCO_2$ | 0.285 | 0.312 | 0.920 | 0.366 | | 2.334 | 0.595 | 3.930 | **0.000** | |
| | Temp × $pCO_2$ | 0.620 | 0.219 | 2.840 | **0.007** | | 1.222 | 0.416 | 2.940 | **0.005** | |
| Growth | Intercept | −3.503 | 1.961 | −1.790 | 0.079 | 0.219 | 0.755 | 0.466 | 1.620 | 0.111 | 0.176 |
| | Temp | −0.044 | 0.015 | −2.960 | **0.004** | | −0.006 | 0.003 | −1.810 | 0.076 | |
| | $pCO_2$ | 0.633 | 0.236 | 2.690 | **0.009** | | −0.060 | 0.054 | −1.110 | 0.270 | |
| | Temp × $pCO_2$ | 0.161 | 0.133 | 1.210 | 0.228 | | 0.096 | 0.038 | 2.550 | **0.014** | |
| Survivorship | Intercept | −0.431 | 1.407 | −0.310 | 0.767 | 0.866 | 1.746 | 1.263 | 1.380 | 0.204 | 0.349 |
| | Temp | −0.062 | 0.011 | −5.810 | **0.000** | | −0.019 | 0.010 | −2.040 | 0.075 | |
| | $pCO_2$ | 0.384 | 0.168 | 2.290 | 0.051 | | −0.026 | 0.151 | −0.170 | 0.867 | |
| | Temp × $pCO_2$ | 0.311 | 0.117 | 2.660 | **0.029** | | 0.018 | 0.105 | 0.170 | 0.867 | |

Please refer to the method section for data transformations, $R^2$ is the overall amount of variance explained by the model, significant effects at the level of $\alpha < 0.05$ are in bold

combined effects from fraction changes of the treatments compared with the control (28 °C, 479 µatm $pCO_2$). Additivity can be presumed when both individual factors are significant, but their interaction is not. In cases where a significant interaction term is present, comparison of the predicted with the observed effect of the combined stressors can reveal if antagonism ($C < A + B$), or synergism is indicated ($C > A + B$; Crain et al. 2008).

All experimental data will be made available via www.pangaea.de.

## Results

Daily temperature and $pCO_2$ measurements inside each aquarium are summarised in Table 1. The actual values

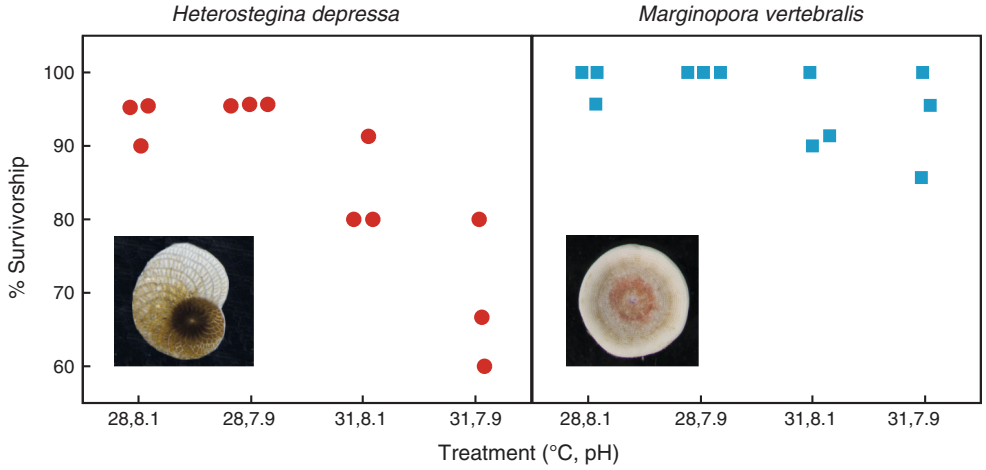


**Fig. 1** Survivorship rates (%) of *H. depressa* (after 35 d) and *M. vertebralis* (after 53 d) at the end of the experiment; data points represent means per aquaria within the treatments. *Inset images* show representative specimens of *H. depressa* and *M. vertebralis* used in the experiment

represent closely the target values of the treatments. The aragonite ($\Omega_{Ar}$) and calcite ($\Omega_{Ca}$) saturation states in the elevated $pCO_2$ (~790 µatm) treatment remained well saturated throughout the experiment, but clearly below that of the 'control' treatment.

Survivorship in *H. depressa* decreased significantly with temperature compared with the controls (Table 2). Survivorship did not decrease with increasing $pCO_2$ (change of 2 %) and decreased significantly in the combined treatment by 26 % (Fig. 1). Both stressors acted synergistically on survivorship in *H. depressa* because proportional change compared with the controls was more than 3 times higher than the predicted additive effect (Table 3). As a result of the high survivorship in *M. vertebralis*, no effects of any of the treatments or their combination on survivorship were found.

Elevated temperature and $pCO_2$ had a significant negative effect on growth in *H. depressa* (Table 2). Although the interaction term is not significant, it is noteworthy that the lowest growth occurred in the combined treatment (Fig. 2). In the control treatment (28 °C, pH 8.1), *H. depressa* grew on average at 0.39 % $mm^2$ $d^{-1}$ which is a factor of five higher than growth rates of *M. vertebralis*, which grew on average at 0.07 % $mm^2$ $d^{-1}$. Elevated temperature and $pCO_2$ alone had no significant effect on growth in *M. vertebralis* but the interaction term was significant (Table 2). The most likely cause for the interaction is a strong increase in growth under elevated $pCO_2$ at 28 °C (49 %) whereas all other treatments were similar to the controls.

In *H. depressa*, temperature had a significant negative effect on all of the photophysiological parameters measured (Fig. 3a–f; Table 2), whereas elevated $pCO_2$ had a significant negative effect on APR and a significant positive effect on EQY. In *M. vertebralis* temperature had a significant negative effect on all three photophysiological parameters whilst elevated $pCO_2$ had no significant effect on either parameter. Significant interactive effects of the treatments have been found in MQY and EQY for *H. depressa* and in EQY for *M. vertebralis* (Table 2). The combined effect of the two stressors on EQY was antagonistic, because the observed inhibition in the combined treatment was lower that the predicted combined inhibition.

For both species, daily average net oxygen production rates were positive in all treatments ranging from 0.03 to 0.05 µg $O_2$ $h^{-1}$ $mg^{-1}$ (Fig. 3g, h). In both species elevated temperatures reduced oxygen production more than respiration rates (µg $O_2$ $h^{-1}$ $mg^{-1}$; Fig. 3g–j) whereas elevated $pCO_2$ levels had no significant effect on production or respiration. A significant interaction of $pCO_2$ and temperature was observed for respiration rates in *M. vertebralis* (Table 2). Compared with the control treatment, respiration rates in the elevated temperature treatment were reduced by 19 % and elevated by 6 % in the $pCO_2$ treatment compared with the controls. The highest reduction in respiration rates (46 %) was observed in the combined treatment, explaining the significant interaction term and highlighting the synergistic effect of both stressors on respiration in *M. vertebralis*.

The chl *a* pigment concentration in *H. depressa* was significantly reduced under elevated temperatures. The interaction with $pCO_2$ was significant as shown by a reduction in the combined treatment by 52 % compared with the controls (Fig. 4). In the elevated temperature treatment it decreased by 41 % and hardly changed (3 %) in the raised $pCO_2$ treatment. The strongest reduction in chl *a* content occurred in the combined treatment, suggesting that the combined effect is synergistic. Chlorophyll *a* pigment content was in the same range in the control

**Table 3** Summary of the significance of individual effects and their interaction based on general linear models given in Table 2 (◇ significant, ● non-significant; $p < 0.05$) and its interpretation based calculations of predicted additive inhibition and fraction changes compared with the controls

| Species | Parameter | Summary of general linear models | | | Observed inhibition compared with the control treatment | | | Predicted inhibition (additive) | Combined effect |
|---|---|---|---|---|---|---|---|---|---|
| | | Temp | $pCO_2$ | Interaction | *A*: Temp | *B*: $pCO_2$ | *C*: Interaction | $A + B-(A*B)$ | |
| *Heterostegina depressa* | MQY | ◇ | ● | ◇ | −0.010 | 0.002 | −0.020 | −0.008 | Synergistic |
| | EQY | ◇ | ◇ | ◇ | −0.112 | 0.004 | −0.064 | −0.108 | Antagonistic |
| | APR | ◇ | ◇ | ● | −0.246 | −0.012 | −0.270 | −0.260 | Additive |
| | Production | ◇ | ● | ● | −0.379 | n.a. | n.a. | n.a. | Only temp effect |
| | Chl *a* content | ◇ | ● | ◇ | −0.414 | 0.032 | −0.517 | −0.368 | Synergistic |
| | Respiration | ◇ | ● | ● | −0.319 | n.a. | n.a. | n.a. | Only temp effect |
| | Growth | ◇ | ◇ | ● | −0.264 | −0.083 | −0.587 | −0.369 | Additive |
| | Survivorship | ◇ | ● | ◇ | −0.105 | 0.022 | −0.264 | −0.081 | Synergistic |
| *Marginopora vertebralis* | MQY | ◇ | ● | ● | −0.023 | n.a. | n.a. | n.a. | Only temp effect |
| | EQY | ◇ | ● | ◇ | −0.273 | −0.042 | −0.235 | −0.327 | Antagonistic |
| | APR | ◇ | ● | ● | −0.412 | n.a. | n.a. | n.a. | Only temp effect |
| | Production | ◇ | ● | ● | −0.436 | n.a. | n.a. | n.a. | Only temp effect |
| | Chl *a* content | ◇ | ◇ | ◇ | −0.406 | 0.080 | −0.724 | −0.294 | Synergistic |
| | Respiration | ◇ | ● | ◇ | −0.187 | 0.060 | −0.463 | −0.115 | Synergistic |
| | Growth | ● | ● | ◇ | 0.079 | 0.489 | −0.140 | 0.529 | Synergistic |
| | Survivorship | ● | ● | ● | n.a. | n.a. | n.a. | n.a. | No sign. effect |

n.a. denotes cases where only a single factor and not the interaction term were significant

treatments before and after the experiment with initial concentrations of 0.13 (SD = 0.03) μg $mg^{-1}$ wet weight and final 0.16 (SD = 0.03) μg $mg^{-1}$ wet weight in *H. depressa* and initial 0.12 (SD = 0.008) μg $mg^{-1}$ wet weight final 0.15 (SD = 0.02) μg $mg^{-1}$ wet weight in *M. vertebralis*. This indicates that symbiont bleaching did not occur in the control treatments.

Temperature, $pCO_2$, and their interaction had significant effects on the chl *a* content in *M. vertebralis* (Table 2). Compared with the control treatment, chl *a* content in the temperature treatment was reduced by 41 %. In the $pCO_2$ treatment chl *a* content was elevated by 8 % compared with the controls. Despite the apparent positive effect of $pCO_2$ alone, the highest reduction (72 %) was observed in the combined treatment, suggesting a synergistic effect of the stressors on this variable.

In summary, due to temperature elevation, 100 % of the parameters in *H. depressa* and 75 % of parameters in *M. vertebralis* were reduced compared with the controls (Table 3). Elevated $pCO_2$ alone had a significant negative effect on 13 % of the parameters among both species but in combination with temperature, 50 % of all measured parameters showed significant negative effects (Table 3).

The strongest reductions in the studied parameters were observed in the treatments where the two stressors acted in combination. An interaction term was statistically significant in half of the measured parameters (Table 2). Of these, 75 % showed a synergistic interaction between the two stressors whereas the remaining 25 % of the interactions were antagonistic (Table 3).

## Discussion

### Photosynthesis, respiration, and chlorophyll *a* content

Elevated levels of temperature had a significant effect on all photosynthetic parameters, which is consistent with results of previous studies of temperature-induced bleaching in benthic Foraminifera (Schmidt et al. 2011; Uthicke et al. 2011). Temperatures above 30 °C appear to lead to damage on the protein level in the holobiont and reduce carbon fixation rates of the symbionts due to reduced expression of the RuBisCO enzyme (Doo et al. 2012). In contrast to the strong effect of temperature, elevated levels of $pCO_2$ had mixed influences on photosynthetic parameters (Table 3). Negative effects of elevated $pCO_2$ on photosynthetic rates of *Symbiodinium* are known to occur at high $pCO_2$ levels, but at intermediate levels, $pCO_2$, even in combination with raised temperature, induced an increase in symbiont oxygen production in *Acropora intermedia* (Anthony et al. 2008).

In our experiment, oxygen production and dark respiration rates were negatively affected by temperature.

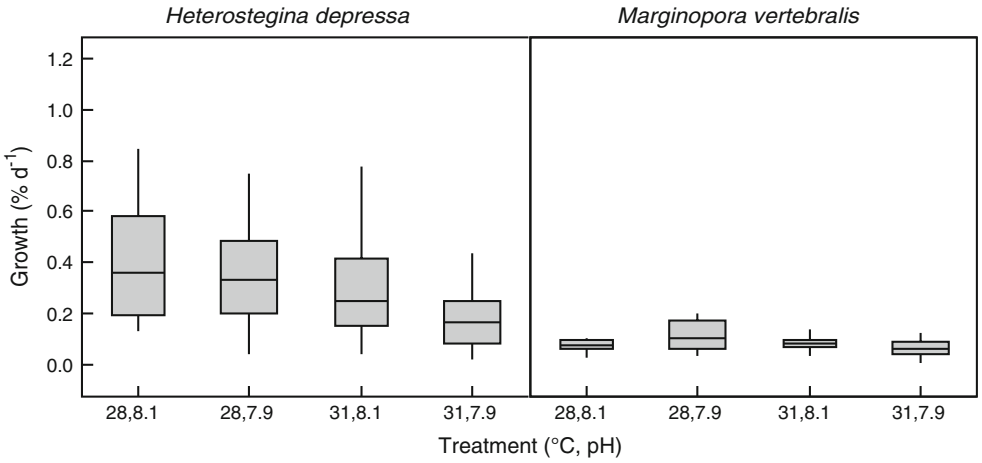


**Fig. 2** Growth rates (% surface area increase $d^{-1}$) of *H. depressa* and *M. vertebralis* during the exposure to experimental conditions for a period of 35 and 53 d, respectively. Data are represented as *box-and-whisker plots*, the *top* and *bottom of the box* (3rd and 1st quartile), the 2nd quartile (median), the *lines* extending from the *box* (*whiskers*) extend to the outermost data that fall within the distance computed as follows: 3rd quartile + 1.5*(interquartile range); 1st quartile − 1.5*(interquartile range)

Previous work suggested that respiration rates in *M. vertebralis* increases with increasing temperatures before and after exposure to 31 °C for several weeks (Uthicke et al. 2011). Despite the fact that an increase in temperature speeds up enzymatic reactions, explaining increased respiration rates in the short term, in the long term, a decline in respiration rates was observed. This shows that the holobiont was weakened in the 31 °C compared with the controls at the end of the experiment. Studies on corals also report reduced respiration rates with increasing temperature (Faxneld et al. 2011; Agostini et al. 2013).

We did not observe any effect of raised $pCO_2$ alone on oxygen production and dark respiration. The result that dark respiration was not affected by $pCO_2$ increase alone, is similar to studies in corals (Langdon et al. 2003; Reynaud et al. 2003). However, our results imply a significant synergistic interaction of the two stressors on the reduction of respiration rates in *M. vertebralis*. The synergistic inhibition of respiration observed in the combined treatment indicates that the metabolic rate of the holobiont is reduced, and this effect may propagate into other vital parameters.

Elevated $pCO_2$ alone increased the amount of chl *a* in *M. vertebralis* but not in *H. depressa*. $CO_2$ fertilisation is known to increase cell numbers, pigments, and productivity in marine unicellular plankton, for example cyanobacteria (Riebesell et al. 1993) and diatoms (Yang and Gao 2012). Productivity increase due to $pCO_2$ increase has also been demonstrated in imperforate Foraminifera (Uthicke and Fabricius 2012) and cell counts of dinoflagellate symbionts in *M. rossi* showed higher symbiont density under elevated $pCO_2$ (Reymond et al. 2013). It is therefore likely that the increased chl *a* content in *M. vertebralis* with $pCO_2$ enhancement in our study reflects higher photosymbiont density. When Foraminifera were simultaneously exposed to elevated temperature, chlorophyll *a* content did decrease significantly, indicating that the hypothesised increase in photosymbiont density due to $pCO_2$ fertilisation was counteracted by temperature, leading to trade-offs in the photosymbiont efficiency and density.

## Survivorship and growth

Elevated temperature reduced survivorship in *H. depressa* but did not affect *M. vertebralis*, indicating that the latter species is more tolerant. Previous work on the temperature response of *M. vertebralis* also showed no effect on survivorship rate under exposure at 31 °C for several months, but reported reduced growth at this temperature (Uthicke et al. 2011). Elevated $pCO_2$ did not influence survivorship in either species, which is similar to previous laboratory studies that reported no significant effect of $pCO_2$ elevation on survivorship in several Foraminifera species (Vogel and Uthicke 2012; McIntyre-Wressnig et al. 2013). However, in *H. depressa* the combined effect of $pCO_2$ and temperature on survivorship was significant and synergistic indicating that this species is especially vulnerable to the combination of stressors.

In our experiment, temperature and elevated $pCO_2$ (738 µatm) in isolation reduced growth in the hyaline species *H. depressa*. In contrast, Vogel and Uthicke (2012) did not report negative effects on growth in *H. depressa* up to $pCO_2$ levels of 1,600 µatm. The reason for these differences is not clear but we note that in our experiment the growth rate of *H. depressa* was higher by almost a factor of two than in the experiments by Vogel and Uthicke (2012).

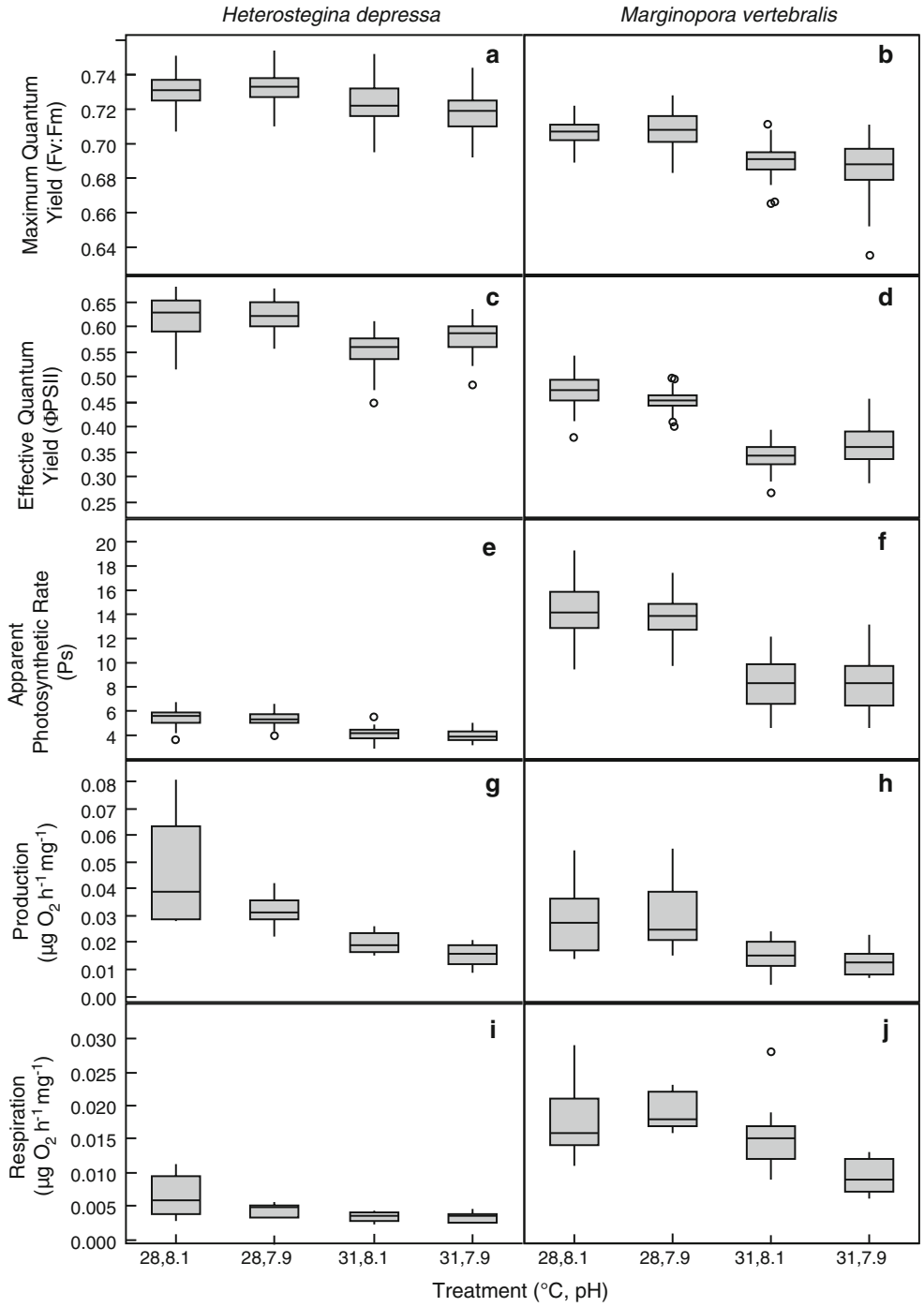


**Fig. 3** **a**–**f** Photo-physiological parameters of *H. depressa* (after 35 d) and *M. vertebralis* (after 53 d) expressed as maximum quantum yield (MQY, $F_v$:$F_m$), effective quantum yield (EQY, ΦPSII), and apparent photosynthetic rate (Ps); **g**, **h** oxygen production (photosynthesis) and **i**, **j** oxygen consumption rates (dark respiration; µg $O_2$ $h^{-1}$ $mg^{-1}$) after exposure to experimental conditions. Explanations of *box* and *whisker* plots are given in Fig. 2, circles present outliers

It is possible that more flow in our system caused higher growth rates in *H. depressa*, leading to significant differences between control and $pCO_2$ treatments in this species (Table 2). Although we have measured growth as an increase in cell volume, it is likely that in both organisms, growth rate is linked with calcification. In this context, we note that flow rates as applied here may have led to a thinning of boundary layers on the surface of the Foraminifera, which may have influenced the scale of local changes of pH induced by photosynthesis and calcification (Glas et al. 2012). A positive influence of water motion on growth rate has been demonstrated in two earlier studies

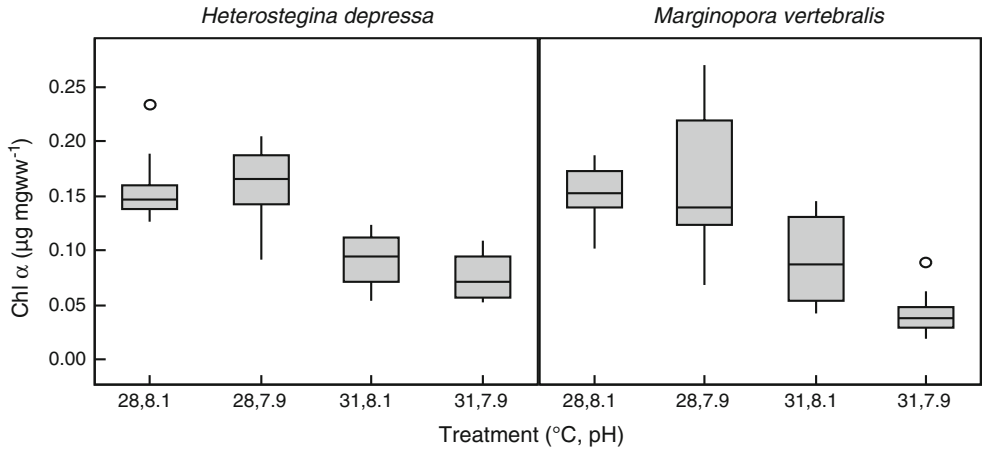


**Fig. 4** Chlorophyll *a* content (µg mg wet weight$^{-1}$) at the end of the experiment in *H. depressa* (35 d) and *M. vertebralis* (53 d) after exposure to experimental conditions, *box* and *whisker* plots are explained in Fig. 2, circles represent outliers

(ter Kuile and Erez 1984; Hallock et al. 1986). On the other hand, the response of growth and calcification in benthic Foraminifera may be decoupled with respect to the effect of $pCO_2$ and the reaction might be species specific. For example, *Amphistegina gibbosa* did not exhibit negative effects on growth of up to 2,000 µatm $pCO_2$, but showed patchy test dissolution (McIntyre-Wressnig et al. 2013). Elevated calcification rates were observed under intermediate $pCO_2$ levels (580–770 µatm) for the clonal population of *Baculogypsina sphaerulata*, whereas the second hyaline species in the same study *Calcarina gaudichaudii*, showed an inconsistent reaction (Fujita et al. 2011). Furthermore, genotype-specific responses to ocean acidification have been reported for other marine organisms, such as coccolithophorids (Langer et al. 2009) and diatoms (Kremp et al. 2012).

In contrast, the porcelanous *M. vertebralis* showed no significant growth response to elevated temperature and $pCO_2$ alone. For this species, growth rates in this study were in the same range as in the previous lower-flow setup (Uthicke et al. 2011; Vogel and Uthicke 2012). A small but not significant increase in growth was observed in *M. vertebralis* in the treatment exposed to elevated $pCO_2$ (738 µatm) at 28 °C. A slight growth enhancement was observed in other porcelaneous species (Vogel and Uthicke 2012), whereas other studies reported reduced growth at medium to high $pCO_2$ levels (700–1,100 µatm) in *Amphisorus hemprichii* (Fujita et al. 2011; Hikami et al. 2011) and *M. rossii* (Reymond et al. 2013).

Differences in the growth and calcification responses to *p*CO2 enhancement are known between and within taxonomic groups (Ries et al. 2009). Varying responses in calcification processes under intermediate scenarios of up to 900 µatm may reflect differential development of protective layers around the precipitated biomineral and different abilities to regulate the pH at the site of calcification (Ries et al. 2009). In our experiment, the moderately elevated $pCO_2$ level acting alone may have released the photosymbiont population of *M. vertebralis* from $CO_2$ limitation (Allemand et al. 2004), which would increase carbon translocation to the host and lead to a growth enhancement.

In all treatments in our study, the $\Omega_{Ca}$ of the seawater remained above two (Table 1) and no test dissolution was observed in any of the treatments. Typically, test dissolution is observed in benthic Foraminifera exposed to much higher $pCO_2$ conditions (2,000 µatm) and at much lower $\Omega_{Ar}$ and $\Omega_{Ca}$ of sea water than in our experiment (Haynert et al. 2011; McIntyre-Wressnig et al. 2013), although at natural $CO_2$ vents, dissolution was observed already at pH ~7.9 (Uthicke et al. 2013). Test dissolution at $pCO_2$ levels equivalent to a pH of 7.4–7.9 was also observed in *M. vertebralis* in laboratory culture (Sinutok et al. 2011), but specimens in the control treatment in that study also showed slight test dissolution; indicating that the cultured Foraminifera were not physiologically fit.

In summary, our results indicate that, at least in the short term, coral reef Foraminifera are likely to continue to grow under conditions predicted for the end of the century. However, the fact that in both studied species, growth was inhibited in the combined treatment indicates that in the long term, growth and by inference calcification under lower saturation may become more difficult. This may ultimately lead to ecological exclusion of these species as observed at present in $CO_2$ seep systems (Dias et al. 2010; Uthicke and Fabricius 2012; Uthicke et al. 2013).

## Combined effects of key global change stressors

Significant interactive effects between $pCO_2$ and warming were observed for 50 % of the parameters investigated. In 75 % of these, for each species, the combined effects were

synergistic—the effects of warming and elevated $pCO_2$ led to a stronger physiological response than the sum of the effects of the individual parameters (Table 3). Because temperature and $pCO_2$ may affect the holobiont as a whole, it is difficult to offer a physiological explanation for the prevalence of the synergistic effects. As a likely explanation, we suggest that the enhanced negative effects of multiple stressors could reflect trade-offs in resource allocation, where the costs of counteracting the effect of one stressor reduce the ability to counteract the effects of the additional stressor. Similarly, it has been suggested that under stressful environmental conditions, such as elevated inorganic nutrient levels, the pressure on the holobiont to control the population size of its photosymbionts might increase, reducing the capacity of the holobiont to respond to stress (Uthicke et al. 2011). The stress caused by overgrowing population of photosymbionts has been described by Wooldridge (2009) to physiologically affect corals and lower their bleaching thresholds. Furthermore, a 3-yr field experiment demonstrated the same effect, where corals bleached more in artificial nutrient enrichment treatments (Vega Thurber et al. 2013).

Interactive negative effects between $pCO_2$ and temperature have been shown to affect the calcification rate in corals, but the response of the corals was highly species specific (Edmunds et al. 2012). In an experiment increasing nutrient levels and elevating $pCO_2$, photosymbiont concentration in *M. rossi* was reduced more under the combined stress than when individual stressors acted in isolation (Reymond et al. 2013). van Dam et al. (2012) showed that populations of Foraminifera exposed to the herbicide Diuron become disproportionately more sensitive to temperature and both factors acted additively on the foraminiferal photosynthetic response. Ecotoxicological studies on the interaction of climate change with additional stressors, such as pesticide exposure, indicate a prevalence of synergistic interactions across different organisms (Holmstrup et al. 2010; Kohler and Triebskorn 2013). In this respect, the observation on the numerous synergistic interactions in our study indicates that the physiology of the unicellular Foraminifera and/or their symbiosis with algae may be affected in a similar way as that of other organisms, contrary to the hypothesis by Pörtner (2002) that protists are less vulnerable to the studied stressors.

Irrespective of the exact mechanism responsible for the existence of strong synergistic effects between the key global change stressors tested in this study, our results indicate that the effects of environmental change in the shallow marine realm under expected $CO_2$ emission scenarios are likely to be underestimated when the effects of elevated $pCO_2$ and temperature are investigated in isolation.

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# Chapter 3

# 3. Publication II: Recent invasion of the symbiont-bearing foraminifera *Pararotalia* into the Eastern Mediterranean facilitated by the ongoing warming trend

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## 3.1. Abstract

The eastern Mediterranean is a hotspot of biological invasions. Numerous species of Indopacific origin appear to have colonized the Mediterranean in historical times, including tropical symbiont-bearing foraminifera. Among these is the species *Pararotalia calcariformata*. Unlike other invasive foraminifera, this species has been discovered only two decades ago and is restricted to the eastern Mediterranean coast. Combining ecological, genetic and physiological observations, we attempt to explain the recent invasion of this species in the Mediterranean Sea. Using morphological and genetic data, we confirm the species attribution to *P. calcariformata* McCulloch 1977 and identify its symbionts as a consortium of diatom species dominated by *Minutocellus polymorphus*. We document photosynthetic activity of its endosymbionts using Pulse Amplitude Modulated Fluorometry and test the effects of elevated temperatures on growth rates of asexual offspring. The culturing of asexual offspring for 120 days shows a 30-day period of rapid growth followed by a slower growth phase. A subsequent 48-day temperature sensitivity experiment indicates a similar developmental pathway and high growth rate at 28°C, whereas an almost complete inhibition of growth was observed at 20°C and 35°C. This indicates that the species may have unexpectedly low tolerance to cold temperatures. We expand this hypothesis by applying a Species Distribution Model (SDM) based on modern occurrences in the Mediterranean and three environmental variables (irradiance, turbidity and yearly minimum temperature). The model reproduces the observed restricted distribution and indicates that the range of the species will drastically expand westwards under future global change scenarios. We conclude that *P. calcariformata* established a population in the Levant because of the recent warming in the region. In line with observations from other groups of organisms, our results indicate that continued warming of the eastern Mediterranean will facilitate the invasion of progressively more tropical marine taxa into the Mediterranean, disturbing local biodiversity and ecosystem structure.

## 3.2. Introduction

Human activities can induce invasions of marine species in two ways: indirectly, by altering the climate and ecosystems (Vitousek et al. 1997), facilitating range expansions (Sorte et al. 2010; Chen et al. 2011), or directly, by mediating species dispersal through anthropogenic means (Carlton 1999).The latter can be realized either as active transport, such as ship traffic, or as removal of barriers to dispersal. The ongoing anthropogenic global change is altering ecosystems at a faster rate than seen in the recent geological past. In consequence, many species are unable to adapt to locally changing conditions through phenotypic plasticity and evolutionary processes and respond by shifting their geographical ranges (Parmesan 2006; Hiddink et al. 2012). This is particularly relevant for marine ecosystems, where species appear to spread an order of magnitude faster than in the terrestrial realm (Sorte et al. 2010). Temperature is the key variable controlling the spread of species and can be used to predict biogeographic range expansions in shallow marine communities (Belanger et al. 2012).

An excellent example of directly mediated invasion of marine species is the opening of the Suez Canal. This event facilitated what is known as the Lessepsian invasion, describing the movement of species from the Red Sea into the Mediterranean after the opening of the Canal in 1869 (Rilov and Galil 2009; Zenetos et al. 2012). If the opening of the Suez Canal was the only factor needed to trigger the range extension of Indopacific species into the Mediterranean, thus all Lessepsian migrants should have appeared near simultaneously. However, the eastern Mediterranean has been experiencing a strong warming trend over the last 20 years (Macias et al. 2013) altering the environmental conditions at the exit of the Lessepsian corridor. The expansion of Indopacific species into the Mediterranean is thus likely exacerbated by climate change and many of the migrating marine species including fish, algae, plants and invertebrates, which continue to expand their range (Hiddink et al. 2012). As a result, the Levantine ecosystem is already dominated by non-native fish species leading to a significant decline of the indigenous populations (Edelist et al. 2013).

A particularly successful group among the Lessepsian migrants are symbiont-bearing benthic foraminifera (Langer et al. 2012; Merkado et al. 2013). The passive dispersal of these organisms appears to be facilitated through the transport of

propagules, which include asexually and sexually reproduced offspring (Alve 1999; Alve and Goldstein 2003). Alternatively, like in many other Lessepsian migrants, the association with marine macro algae represents another means of passive dispersal of benthic foraminifera into the Mediterranean (Zenetos et al. 2012; Caruso and Cosentino 2014). Also the introduction of foraminiferal species in new habitats via ballast waters has been documented (McGann et al. 2000). Symbiont-bearing foraminifera have well defined, species-specific temperature tolerances (Langer and Hottinger 2000). Temperatures exceeding the upper thermal threshold cause symbiont bleaching (Schmidt et al. 2011), whilst low temperatures prevent the establishment of populations (Langer and Hottinger 2000). The symbiosis in benthic foraminifera provides an energetic advantage (Hallock 1981), with the photosymbiont having a double role, by providing nutrition (Lee and Hallock 1987) and promoting calcification (de Nooijer et al. 2009). Symbiont-bearing foraminifera are thus ideally adapted to the oligotrophic conditions of the eastern Mediterranean (Sisma-Ventura et al. 2014). Indeed, the appearance of invasive symbiont-bearing foraminifera in the eastern Mediterranean is documented at least since the 1960s (Langer 2008) and their ongoing proliferation has significant impact on coastal ecosystems (Mouanga and Langer 2014).

The most recently described migration of symbiont-bearing benthic foraminifera into the Levantine basin involves a species of the Indopacific genus *Pararotalia*. This species has been first reported in the Levant in 1994 by Reinhardt et al. (1994) and Yanko et al. (1994). It has since then been found to proliferate along the Mediterranean coast from Israel (Hyams-Kaphzan et al. 2008; Arieli et al. 2011) to southern Turkey (Meriç et al. 2013). The modern foraminiferal fauna of the Mediterranean Sea is mostly of Atlantic origin (Langer 1993; Langer 2008). After the opening of the Suez Canal in 1869, many tropical symbiont-bearing foraminifera migrated into the Mediterranean Sea, including amphisteginids, soritids, and heterosteginids (Hyams et al. 2002). The apparently later invasion and more restricted occurrence of *Pararotalia* contrasts with other symbiont-bearing foraminifera, implying that the invasion of *Pararotalia* was not facilitated solely by the physical connection of the Suez Canal. We note that the invasive *Pararotalia* is not a “classical” Lessepsian species, as it has not yet been found in the Red Sea (Reiss and Hottinger 1984; Parker et al. 2012; Madkour 2013). However, with the exception of the Gulf of Aqaba (Hottinger L et al. 1993), the diversity of foraminifera in the Red

Sea is not well known and considering the habitat of the species and its distribution in the Indopacific, it is likely that it also occurs in the Red Sea (Hesemann 2014).

To understand the explosive recent invasion of *Pararotalia* in the Mediterranean, we carried out an investigation of the ecology and physiology of this species. We investigated its genetic relationship with Pacific populations of the genus, identified its endosymbiotic microalgae, determined its photosynthetic activity from freshly collected specimens and monitored their photosynthetic activity in the laboratory cultures. We hypothesized that like in other foraminifera (Goldstein and Alve 2011) and in many other marine species (Belanger et al. 2012) temperature is the main factor controlling the establishment of new populations. Therefore, we carried out an experiment exposing asexual offspring of *P. calcariformata* originating from the invasive population in the Mediterranean to three temperatures (20°C, 28°C and 35°C) to determine their survival and growth rates under these conditions. Using a compilation of all occurrence records of the species in the Mediterranean, we model its likely spread under future climate change.

## 3.3. Material and Methods

### 3.3.1. Sample collection and maintenance of cultures

Living specimens of *Pararotalia calcariformata* were collected during four field campaigns on 23/10/2012, 01/11/2012, 08/04/2013 and 14/04/2013 at Nachsholim, Israel (32°37'25.0"N, 34°55'11.4"E) at 0.5-2m. From Hadera, Israel samples were obtained from two shallow locations at depths of 0.5-1m (32°27'40.9"N,34°52'57.0"E and 32°26'49.1"N, 34°52'40.8"E) and at two deep locations from 5-7m (32°27'40.0"N, 34°52'46.4"E and 32°27'15.9"N, 34°52'35.5"E). Samples were collected by sampling macroalgae substrate (either turf algae or the filamentous coralline algae *Jania* sp.*)* by snorkeling or SCUBA diving. Samples were transported in large plastic bottles filled with algae and sediment to the laboratory, where the algae and sediment was rinsed with sea water and specimens were picked from the concentrated sediment and put to a maximum number of 50 specimens in screw capped plastic jars (volume 100 mL). The jars were shipped inside an insolation container to Germany (express shipment time 48 h). The specimens were cultured in plastic containers at in-situ sea water temperatures (23-24°C in December, 20-21°C in April) under a diurnal 12 h/12 h light cycle, salinity 38.5-40 ‰ and irradiance of <30 µmol photons $m^{-2}$ $s^{-2}$. The

seawater was prepared from artificial sea salt (Tropic Marin® Sea Salt, Germany) every two weeks. Approximately 50% seawater was exchanged weekly and the cultures were fed with *Nannochloropsis* food mixture every 6-8 weeks. Food mixture contained algae concentrate (12 x $10^9$ cells/mL, BlueBioTech GmbH, Germany), which was diluted with artificial seawater (30 µL *Nannochloropsis* concentrate: 200 mL artificial seawater) and autoclaved. The sample of *Pararotalia* sp. from Pelican Island, Australia was collected on the 05/05/2014, sent by express shipment to Bremen and cultured under the above conditions and salinity of 35-36 ‰ prior to DNA analysis.

### 3.3.2. Taxonomic identification and habitat

The morphology of *P. calcariformata* from the Israeli coast has been documented using SEM (Scanning electron microscopy) and light microscopy with a digital camera (Leica, DFC290HD) (Fig. 1). A detailed taxonomic description of *P. calcariformata* is provided in Supporting Information S1, including SEM images showing adult and juvenile stages. All samples originated from Nachsholim and represent specimen grown under natural conditions. Occurrence records in the Mediterranean were obtained by literature search and own sampling and compiled (shown in Fig 6). We also carried out a literature search for *Pararotalia* morphological forms named as or resembling *P. calcariformata* in the Indopacific (shown in color map Fig. 2).

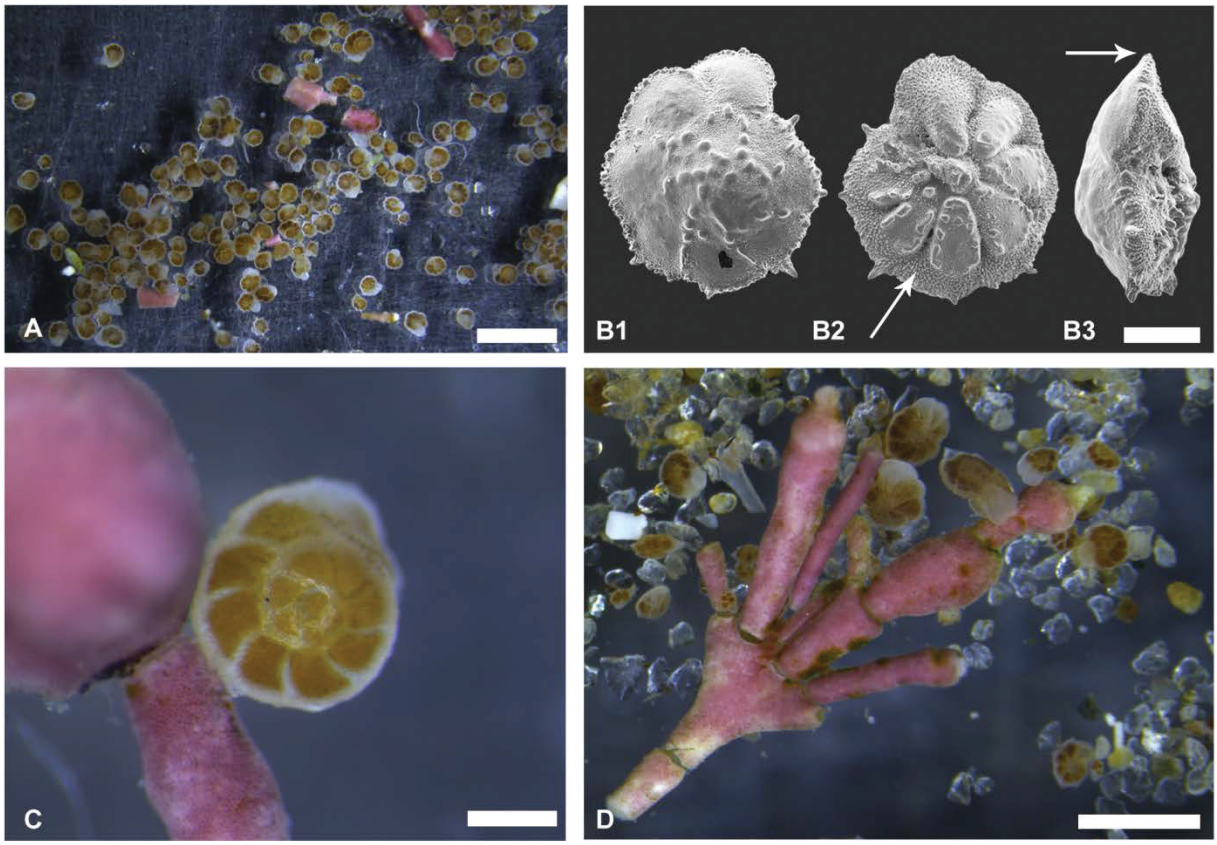


**Figure 1. Shell morphology and live appearance of *Pararotalia calcariformata* from Nachsholim, Israel, collected in April 2013. A) Light microscope image of a natural population, scale bar 2 mm. B) Scanning electron images of a representative specimen in spiral, umbilical and lateral views, arrows indicate septal interlocular spaces (B2) and keel (B3), features supporting the identification of the species in line with the original description by McCulloch (1977), scale bar 100µm. C) and D) Light microscopy images of specimens attached on *Jania rubens* coralline algae, scale bar in C) 200 µm, in D) 1 mm.**

### 3.3.3. Symbiont culturing and preparation for SEM microscopy

We have isolated and cultured the symbionts of five specimens of *P. calcariformata* collected in Nachsholim, Israel on 23/10/2013. The specimens were taken from cultures collected on 8/11/2013 and the isolates were grown in standard culture media (Guillard's (f/2) Marine Water Enrichment Solution, Sigma Aldrich) (Lee et al. 1979; Lee and Anderson 1991). Cultures were terminated after 4 weeks of growth, oxidized by $H_2O_2$, filtered onto Nucleopore Track Etch Polycarbonate filters (Whatman) that were cut to fit the size of the metallic stubs used for examination in the SEM.

### 3.3.4. PAM Fluorometry

To prove that *P. calcariformata* establishes a permanent photosymbiosis with algae we characterized its photosynthetic activity by Pulse Amplitude Modulated (PAM) fluorometry. For measurements of dark adapted yield (maximum quantum yield, MQY, $F_v$:$F_m$) of Photosystem (PS) II, IMAGING-PAM M-Series fluorometer (IPAM, WALZ GmbH, Germany) was used. It was equipped with MAXI-Head, 1/2'' CCD camera and zoom objective (F1.0/f=8-48 mm). Specimens were transferred in Petri dishes containing fresh seawater and dark-adapted 10-15 minutes before measuring $F_v$:$F_m$. We elevated the Petri dishes closer to the zoom objective on a 1.5 cm high stand and used the Leaf Holder IMAG-MIN/BK to allow best possible imaging for all specimens (size 0.3-0.4 µm). Other procedure followed the protocols previously published (Hill et al. 2004; Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014).

Using the IPAM we also measured rapid light curves (RLC) to access photosynthetic activity in the symbionts in *P. calcariformata* (Ralph and Gademann 2005) under different light intensities. After each light intensity step, the effective quantum efficiency (Y(II)) of the symbionts is automatically measured by the Imaging Win Software (WALZ, Germany). Dark adaptation was chosen to be for 10 s as in Ralph and Gademann (2005) followed by several increasing light intensities for 10 s each. The irradiance steps emitted by the LEDs of the IPAM instrument were calibrated using a hand-held PAR Light Meter (Apogee, USA) and were as follows: 0, 11, 26, 42, 65, 92, 125, 164, 213, 264, 313, 385, 450, 534, 604, 682, 780 µmol photons $m^{-2}$ $s^{-1}$. For every irradiance level, the relative electron transport rate (rETR = E × Y(II)) was calculated and rETR versus Apogee PAR light intensity (E) were plotted (Fig. 4A). Several photosynthetic parameters were drawn in the curve for illustration such as $ETR_{max}$ (maximum height of the curve), the $E_k$ (minimum saturating irradiance level) and the slope of the curve (α), which aid in determinging the photosynthetic activity level. However, as we do not intent to compare the response between different light levels or species, we simply estimated them based on a standard curve (cubic spline with a default lambda of 0.05) fitted by Jmp 11 (SAS 2014). Measurements on 17/04/2013for the RLC were conducted on 30 randomly selected specimens three days after sampling, pre-adapted to light levels <30 µmol photons $m^{-2}$ $s^{-1}$ levels.

### 3.3.5. Reproduction and offspring experiments

To test whether the asexual offspring can be cultivated under laboratory conditions we randomly choose six juveniles that were found in the culture in December 2012 and monitored their development for 117 days (12/2012 – 04/2013). To this end we used a 6-well plate filled with artificial seawater (~15 ml volume per well), closed with a lid and placed inside an incubator illuminated at 10-15 µmol photons $m^{-2}$ $s^{-1}$ (Leec Plant Growth Cabinet, Model PL2, 150 litre, UK), as tested in previous work (Schmidt et al. 2011). *Nannochloropsis* food mixture (see general culturing) was added to each well on11/12/12, 22/12/13, 11/01/13 and 08/03/13. The juveniles were transferred to a new plate before each feeding to ensure the same conditions in each well. Water was exchanged by 50% twice weekly and the specimens were photographed and measured. Salinity was monitored with a handheld salinity and temperature meter (WTW and Oregon Scientific, USA) before each water exchange. Salinity stayed between 38.9-39.3 ‰ during the culturing period, simulating natural conditions. Temperature of the incubator was adjusted to natural winter conditions in the Levant during the culturing period (for 12/12 from 23-24°C followed by a slightly colder period from 01/13- 03/13 (20-22°C).

Following the initial culturing of a small number of asexual offspring, we tested the development of the asexual offspring under different temperatures (20, 28, and 35°C) in a shorter and replicated design using 54 individuals. These were randomly selected from ~400 juveniles from the May 2013 reproduction event and cultured for 48 days (05/2013- 07/2013). Three thermostatic cabinets (Pol-Eko-Aparatura, Model ST2+/ST2+, Poland) were used for the setup containing three 6-well plates each with 18 specimens exposed to the same illumination level in the incubator. All plates received the same light conditions (12-hour cycle, 25-30 µmol photons $m^{-2}$ $s^{-1}$) throughout the experiment. To ensure that growth was not influenced by the location of the plate inside the incubator, the order of the plates was changed randomly when the water was exchanged three times weekly. Light levels were chosen to be higher than in the previous culturing work because rapid light curves (RLC) from 04/2013 indicated better performance of the photochemistry of the symbionts (Fig. 4A) under slightly elevated conditions. *Nannochloropsis* food mixture (see general culturing) was added to each well at the onset of the experiment at 18/05/13 and at 19/06/13. A more frequent feeding was not necessary as enough food was still visible in the

plates. Water exchange was performed three times weekly and manual monitoring of salinity and temperature was done in the same way as described before. At the onset of the experiment temperatures in incubators were slowly ramped up to prevent acute temperature stress. The 28°C and 35°C incubator were automatically ramped up to final temperature over a period of 12 h (2°C every 2-3 h). Manual temperature measurements (n=57) conducted inside the plates showed that actual temperatures in the incubators varied minimally 20.3°C (SD 0.1°C), 27.7°C (SD 0.2°C) and 34.7°C (SD 0.3°C). During the first 72 h of the experiment salinity peaked by +2-4 ‰ above the target levels at 38.5-40‰, which otherwise remained constant inside the wells throughout the experiment (Mean 39.7 ‰, SD 1.1). This short time span of elevated salinity did not cause mortality or bleaching, as the species has been shown to inhabit high saline areas (Reinhardt et al. 2003) and is generally adapted to very saline water of the Levant (Herut et al. 2000). The pH of the artificial ocean water was monitored weekly with a handheld meter (WTW, Germany) and stayed between 8.1-8.2 units.

### 3.3.6. Growth measurements

Twice weekly during culturing experiments specimens were photographed inside each well (Fig. 5B) with an inverse microscrope (Zeiss PrimoVert) and images were taken at the resolution of 5184 x 3456 pixel (Canon SLR camera). Largest diameters were measured starting at the last build chamber diagonally across the shell, using image analyzing software (ImageJ). Growth rates (% diameter increase day-1) were calculated using the formula given in Schmidt et al. (2011) modified from ter Kuile and Erez (1984).

At the end of the temperature sensitivity experiment 46 of initially 54 could be retrieved for measurements (1/18 missing in 20°C, 3/18 in 28°C, 4/18 in 35°). Although, the shell diameter increased in most specimens throughout the experiment (=positive growth, indicated in % in Fig. 5 A), we observe apparent negative growth rates in 35% of the specimens in the 20°C treatment (6 out of 17 specimens) and 36% in the 35°C treatment (5 out of 14 specimens). The negative growth reflects measurement uncertainty due to uneven orientation and attached debris, and is interpreted to indicate lack of growth. To exclude the possibility that the observed positive growth rate also represent measurement uncertainty, the total number of

chambers was counted for specimens where the shell was best exposed. In both the 20°C and the 35°c treatments, we could identify specimens that added at least one chamber during the experiment. One-way Analysis of Variance (ANOVA) was used to test the effect of temperature on growth rates of asexual offspring. Statistics were performed on all individuals exhibiting positive growth rates (Fig. 5 A). Growth data was 3$^{rd}$ root transformed and residual and normality plots indicated that equal group variance was not violated. ANOVA was performed using Jmp 11 (SAS 2014).

### 3.3.7. DNA extraction, amplification, cloning and sequencing

Three specimens of *P. calcariformata* collected from Hadera Ridge, Israel (C50) and in Nachsholim, Israel (C228 and C229) on 23/10/2013 and one specimen of *Pararotalia sp*. collected in Pelican Island, Australia (C538) on 05/2014 were isolated from cultures into 50 µl of GITC* on 07/11/2013 and 26/05/2014. Because of the thickness of the shell, the specimens were crushed with a metal rod prior to DNA extraction. A fragment of ~1000 bp located at the 3' end of the SSU rDNA of the foraminifer was amplified using the primer couple S14F1 (5'-AAGGGCACCACAAGAACGC-3') – 1528R (5'-TGATCCTTCTGCAGGTTCACCTAC-3') by Polymerase chain reaction (PCR) (Medlin et al. 1988; de Vargas et al. 1997) using the GoTag (Promega, USA) or PHUSION (Thermo Scientific, USA) polymerase. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Netherlands) and cloned with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA) with TOP10 chemically competent cells following manufacturer's instructions. Three to six clones were sequenced per individual by an external provider (LGC Genomics, Berlin).

A fragment of ~400 bp of the 3' end of the SSU rDNA of foraminifers' symbionts was obtained from aliquots of the same DNA extractions using the GoTaq polymerase (Promega, USA) with the symbionts specific forward primer SymSF1 (5'-GGTTAATTCCGTTAACGAACGAGA-3') coupled with the universal reversed primer 1528R (5'-TGATCCTTCTGCAGGTTCACCTAC-3') for the specimen C228 and C229. No multiple bands have been observed after migration of the PCR product on agarose gel. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Netherlands) and directly sequenced. The sequence chromatograms were carefully checked and no sign of multiple signals was detected.

### 3.3.8. Sequence analysis

The obtained 19 sequences of *P. calcariformata* were analysed together with 24 sequences of benthic foraminifera belonging to the lineage of *Globothalamea* (Pawlowski et al. 2013) downloaded from GenBank (Table S3). The sequences were automatically aligned with MAFFT v.7 (Katoh and Standley 2013) with default options. Only the fragment covered by the obtained sequences of *Pararotalia* was retained for further analyses (see alignment in Table S3). The model of evolution (GTR+I+G) was selected using jModeltest v. 2.1.4 (Darriba et al. 2012) under Akaike Information Criterion (AIC). Using this model of evolution, the most likely tree topology was inferred from the alignment using a Maximum Likelihood Approach implemented in PhyML 3.0 software (Guindon et al. 2010), using NNI+SPR tree improvement and non-parametric bootstrapping (1000 pseudo replicates). The resulting tree was visualized with iTOL v 2.1 (Letunic and Bork 2011, Fig. 2). The two symbiont sequences were compared to the SILVA database (Yilmaz et al. 2014) on the 21/08/2014 in order to determine their most probable taxonomic assignation. The SINA 1.2.11 alignment tool (Pruesse et al. 2012) has been used with default options.

### 3.3.9. Computation of the habitat model

Occurrence records of *P. calcariformata* in the Mediterranean were obtained by literature search and combined with new observations during this study (shown in Fig. 6). For the calibration of the species distribution model (SDM), occurrences were converted to presence records on a grid used by the modeling software. Environmental data for these grid cells were obtained from the BIO-Oracle database, which provides oceanographic variables with a grid-cell resolution of 5 arcminutes (Tyberghein et al. 2012). BIO-Oracle also includes gridded data from climate model projections that are based on SRES climate-change scenarios (Jueterbock et al. 2013) and for our model we used the intermediate scenario A1B for the a projection to year 2100. We based the SDM for *P. calcariformata* mainly on temperature (annual minimum SST) and added annual mean diffuse attenuation (mean DA) and annual mean photosynthetically available radiation (mean PAR). The latter variables provided the possibility to incorporate the effects of terrestrial and trophic influences, as well as solar radiation on the potential distribution. These variables have been proven useful in previous modeling calibrations from other symbiont-bearing

foraminifera (e.g. Langer et al. 2013). The resulting SDM was refined in a two-step clipping process in order to avoid a biased relation between the variables, an approach that has been successfully used in other models on foraminiferal distributions (Weinmann et al. 2013a). First, we used only minimum SST, which was subsequently projected on the future climate scenario. Second, we built a model on mean DA and mean PAR. The final SDM for both current and future conditions (Fig. 6) thus comprises a climate-model based on temperature (including the projection on the A1B scenario), which was overlain and clipped by a habitat-model based on the other variables. The editing of the climate model was performed with the software DIVA-GIS.

We used Maxent 3.3.3k (Phillips et al. 2006) to model the potential distribution of *P. calcariformata* in the eastern Mediterranean and to project it onto future climate conditions. The program uses a grid-based machine-learning algorithm following the principles of maximum entropy (Jaynes 1957). In the course of the modeling process, Maxent begins with a uniform distribution and successively fits it to the data (occurrence records and environmental variables). For an overview on the operating mode of Maxent and the interpretation of its output see Elith et al. (2011). Note that Maxent does not predict the actual distribution of the taxon, but rather the relative suitability of the habitat, which is interpreted as the potential distribution of the taxon under study. A total of 10,000 random background points were automatically selected by Maxent within the eastern Mediterranean. The logistic output format with suitability values ranging from 0 (unsuitable) to 1 (optimal) was used (Phillips and Dudik 2008), where the probability of presence at sites with "typical" conditions is set to 0.5 by default (Elith et al. 2011). The modeling process was performed with 50 replicates and the average predictions across all replicates were used for further processing. The continuous probability surfaces of the SDMs were subsequently converted into presence/absence maps using the “Equal training sensitivity and specificity logistic threshold” as recommended by Liu et al. (2005), which has also been used in previous foraminiferal models (see Langer et al. 2013).

Projecting a model on future climate scenarios may result in an extrapolation or “clamping” of the probability values (Phillips et al. 2006) especially in regions where the environmental predictors are outside the training range, which could lead to an over- or underfitting of the model. In Maxent, a multivariate similarity surface (MESS)

analysis is implemented, which shows how similar predictor variables within future climate scenarios are seen during model training (Elith et al. 2011). We added the result of the MESS analysis to our future model, highlighting areas of possible extrapolation of the model due to minimum temperature values of the future scenario being outside the training range.

## 3.4. Results and Discussion

### 3.4.1. The identity of the invasive species and its current distribution

*Pararotalia calcariformata* McCulloch 1977 has been only very recently added to the list of over 700 marine species that appear to have invaded the Mediterranean in historical times (Zenetos et al. 2012). The species has been reported from littoral environments of the Israeli coast as *Eponides* by Yanko et al. (1994) and as *Pararotalia spinigera* by Reinhardt et al. (1994). The earliest description in the Levant of the genus *Pararotalia* is by Reiss et al. (1961), who reported in 1961 on the occurrence of *Pararotalia cf. ozawai* (Graham & Millitante (non Asano)) from a locality near Haifa. Unfortunately, he provides no illustration and the material of the collection is not available. Therefore, we cannot confirm this record and conclude that the exact time of introduction of the species cannot be constrained beyond occurring prior to 1994. However, we note that the species must be a very recent invader, as the study by Hyams-Kaphzan et al. (2014) found specimens attributed to *Pararotalia calcariformata* only in surface sediments and never in historical layers. Other studies confirmed the proliferation of the species after 1994, identifying large populations ranging from the southernmost Israeli coast (Hyams-Kaphzan et al. 2008; Arieli et al. 2011) to southern Turkey (Meriç et al. 2013). All localities where this species has been reported up to now in the literature as well as through our investigations in the eastern Mediterranean are given in Figure 6.

Considering the large abundance of the species and its distinctive shape, it is unlikely that it has been overlooked in earlier studies or not seen at other localities in the eastern Mediterranean (Meric et al. 2008; Langer et al. 2012). Collectively, the existing evidence implies that the species has established the Levant only recently and began to proliferate and expand its range within the last two decades (Meriç et al. 2013). The majority of the invasive species in the eastern Mediterranean represent Lessepsian migrations through the Suez Canal (Zenetos et al. 2012). The invasive *Pararotalia* has not yet been described from potential source locations in the Red Sea such as the Gulf of Aqaba (Reiss and Hottinger 1984), the Gulf of Suez (Madkour 2013) or the South Sinai Coast (Parker et al. 2012). On the other hand, *Pararotalia* appears to be an Indopacific genus (Langer and Hottinger 2000) and specimens assignable to *P. calcariformata* have been found along the coast of the

Arabian Sea, Oman. Therefore, it is most likely that *P. calcariformata* also followed the Lessepsian route. Under this scenario, the species could have invaded the Mediterranean across the Suez Canal, for example attached to gastropod larvae (Cedhagen and Middelfart 1998). Alternatively, it could have been introduced with ballast waters, as has been hypothesized for the red alga *Grateloupia yinggehaiensis* possibly introduced to the Mediterranean from China by ship traffic (Wolf et al. 2014). This alga appears to have established a continuous population in the vicinity of a thermoelectric power plant (Wolf et al. 2014). We note that *P. calcariformata* has been found to occur prolifically in the heat plume of the thermal power plant in Hadera (Arieli et al. 2011), which might also have has served as a stepping stone for its invasion. The exact origin of *P. calcariformata* and its dispersal route can only be established by the identification of a potential parent population, such as it has been shown for the invasive *Sorites* by Merkado et al. (2013), who found a potential parent population in the Gulf of Aqaba. Therefore, we conducted a taxonomical and genetic investigation to characterize the relationship between the Indopacific and Levantine populations of *Pararotalia*.

The morphological taxonomy of the invasive *Pararotalia* population has been confusing, because the first specimens found and identified in the Mediterranean were mistakenly classified as *Eponides repandus* (Fichtel and Moll, 1798) (Yanko et al. 1994) and only later assigned to *Pararotalia spinigera* Le Calvez 1949 (Bresler and Yanko 1995a,b). The generic classification has been stable since, but the species level taxonomy within this morphologically variable genus is in need of revision. Previous studies by e.g. Reinhardt et al. (1994); Hyams-Kaphzan et al. (2008); and Arieli et al. (2011) designated recent specimens from the Israeli coast as *Pararotalia spinigera* Le Calvez 1949, following Loeblich and Tappan (1987) and Hottinger et al. (1991). Meriç et al. (2013) first suggested that the specimens may rather represent *P. calcariformata*, an extant species described from the Indian Ocean by McCulloch (1977). *Pararotalia calcariformata* McCulloch 1977 is mainly distinguished from *P. spinigera* (Le Calvez 1949) as described by Loeblich and Tappan (1987) and Hottinger et al. (1991) in having a distinct peripheral keel and deep septal interlocular spaces on the umbilical side (Fig. 1B2-3). A comparison of the morphology of the Levantine species with this description confirms that the assignment of the invasive species to *Pararotalia spinigera* is incorrect, because a keel and deep septal interlocular space are clearly present among the Levantine

specimens. Our analysis also confirms that the specimens we examined have similar appearance as those reported by Meriç et al. (2013) from Hatay, Turkey. Therefore, we here conclude that morphologically, the invasive species shows most affinity with the concept of *P. calcariformata* and we use this name henceforth when referring to the extant populations from the Levant.

Having established its likely taxonomic identity and the range of morphologies represented in the Levantine populations (Supporting Information S1), we next assessed the distribution of its potential parent populations. The species *P. calcariformata* was originally described by McCulloch (1977) from recent shallow habitats in Australia and Ceylon, and later reported throughout the Indopacific. In addition, specimens closely resembling *P. calcariformata* have been identified from among SEM images available through the Foraminifera.eu project (Hesemann 2010) in faunas from Australian beaches, Malaysia, Oman and Iran. These observations indicate that the species is a common element of tropical and subtropical assemblages throughout the Indopacific (Fig. 2).

To confirm the Indopacific origin of the Levantine population using genetic inference, we have obtained SSU rDNA sequences from specimens collected from Israeli coast and specimens of a morphologically similar species of *Pararotalia sp.* from Australia and compared these with published sequences of *Pararotalia nipponica* originating from Japan. The relationship among these species was assessed by constructing a phylogeny rooted on agglutinated foraminifera and including representative sequences of all major calcareous clades, as presented in Pawlowski et al. (2013) .The resulting phylogenetic tree (Fig. 2) has an almost identical topology to that inferred by Pawlowski et al. (2013), although the branch support is lower, most probably due to a shorter sequence length of the analyzed SSU fragment. All the obtained sequences of *Pararotalia* form a highly supported monophylum. The Levantine population sequences cluster within one clade and appear more closely related to sequences belonging to *Pararotalia nipponica.* This suggests that, the Levantine population is derived from within the Indopacific radiation of the genus. The Australian *Pararotalia sp.* is similar to *P. calcariformata* in possessing spines, but these are much more regularly developed, such as in the species *P. stellata* (de Férussac, 1827). A clarification of the relationship among the three studied forms would require a comprehensive taxonomic revision of the group.

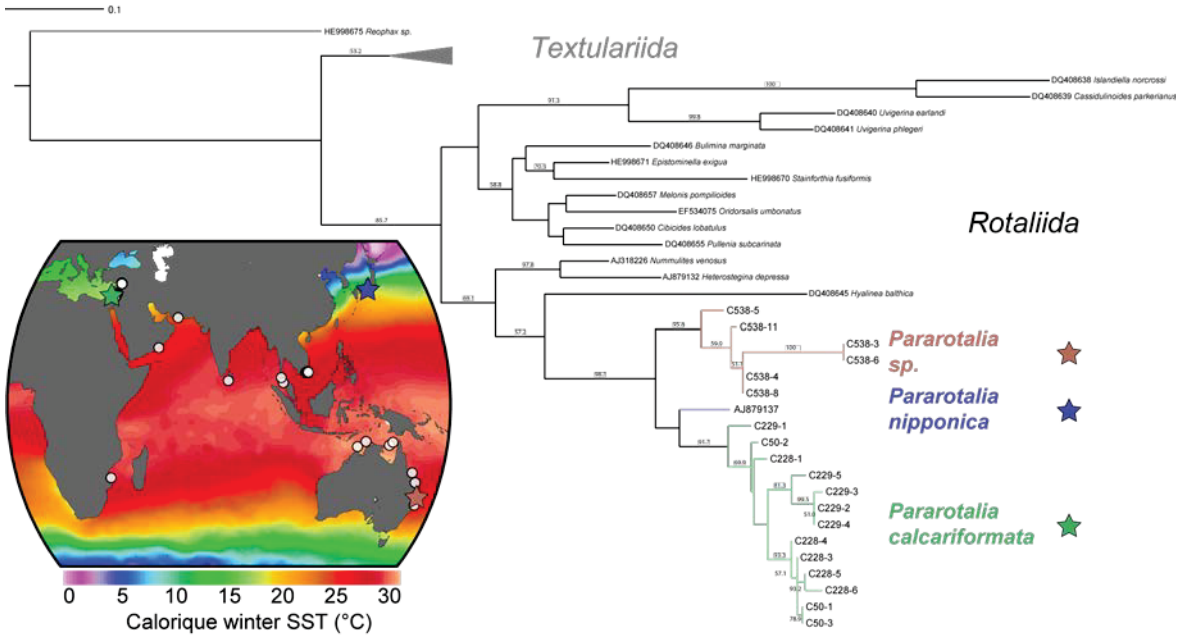


**Figure 2. Phylogenetic tree (Maximum Likelihood, GTR+I+G) showing the evolutionary relationships of *Pararotalia* with other benthic foraminifera belonging to the order Globothalamea. Bootstrap scores (1000 replicates) higher than 50% are shown next to the branches. The tree is rooted on the genus *Reophax*. The occurrence of the genus in the Indo-Pacific is shown on the map against winter sea surface temperature (data extracted from the World Ocean Atlas 2005 Locarnini (2006). Open circles represent the occurrences of *Pararotalia* reported in the literature and stars denote the location of the sequenced specimens.**

### 3.4.2. Characterization of diatom endosymbionts and their photochemistry

The ecology of symbiont-bearing benthic foraminifera is closely tied to the function of their algal symbionts. Although the genus *Pararotalia* together with the genus *Neorotalia* is considered to belong to the informal group of larger benthic foraminifera (LBF), which are typically associated with algal symbionts (Langer and Hottinger 2000), the presence and identity of the symbionts in *Pararotalia* has never been formally established. In addition, the Mediterranean *Pararotalia* is smaller (typically <400 µm) than other symbiont-bearing benthic foraminifera. One study reporting the occurrence of the invasive *Pararotalia* inside a thermally polluted site along the Israeli coast (Arieli et al. 2011) noted a distinct coloration of its cytoplasm, which is often an indicator for the presence of algal symbionts. Therefore, prior to further physiological experiments, we investigated the presence and nature of symbionts in the studied Levantine population.

In molecular phylogenies, *Pararotalia* appears to cluster with Calcarinidae and Nummulitidae (Schweizer et al. 2008) which are typically associated with diatom symbionts (e.g. Lee and Anderson 1991; Lee et al. 1995; Lee and Correia 2005). However, the diatom symbiosis in foraminifera is known to involve different, potentially multiple species (Lee 1995), prompting us to use two methods to determine the identity of the symbionts in the investigated Levantine population. First, we isolated the symbionts by crushing the calcite shell, opening the protoplasm and growing the cellular content in axenic media. Endosymbiotic diatoms extracted and grown in this manner begin to produce frustules, allowing their morphological classification (Lee et al. 1979). Symbiont cultures obtained from five specimens of *P. calcariformata* from the locality Nachsholim revealed, in three cases, the presence of multiple species of diatoms and in two cases only a single diatom species. In four cultures the diatom could be identified as *Minutocellus polymorphus* (Hargraves & Guillard) Hasle, Stosch, & Syvertsen (Fig. 3). In three cultures *Navicula* sp. was observed, whereas *Amphora bigibba* and *Amphora* sp. (with asymmetrical raphe) were only observed in one culture each.

The results of the symbiont culturing show that several species of diatoms can be identified within the same host. This is in line with previous work on related taxa by Lee et al. (1995). So far, 20 diatom species or varieties have been isolated as potential symbionts of foraminifera (Lee 1992). Of these, *Nitzschia frustulum* var. *symbiotica* (Lee et al. 2000) is the most commonly isolated diatom endosymbiont (Lee 2006). Interestingly, we did not find this species in the cultures derived from *P. calcariformata*. In contrast, the most commonly identified potential symbiont *Minutocellus polymorphus* has never been observed in a benthic foraminifera host before (Lee 2006). This diatom is found free-living in the Mediterranean (Sarno et al. 1993) and given its small size (up to 3 µm) it can plausibly act as a symbiont.

To confirm that this species was numerically abundant during life of the *P. calcariformata* holobiont, we amplified a SSU rDNA fragment of total DNA extractions from two specimens from the Nachsholim locality. The amplification was carried out using primers that were designed to anneal with a range of eukaryotic lineages but not the foraminifera host (see methods). In both specimens, the PCR (Polymerase chain reaction) product yielded a single electrophoresis band, which could be directly sequenced, indicating the presence of a numerically dominant signal. The resulting

sequences could be unambiguously identified as *Minutocellus* by comparison with the SILVA database (Yilmaz et al. 2014). Therefore, we conclude that the diatom endosymbiont consortium in the investigated specimens of *P. calcariformata* was likely dominated by *M. polymorphus.*

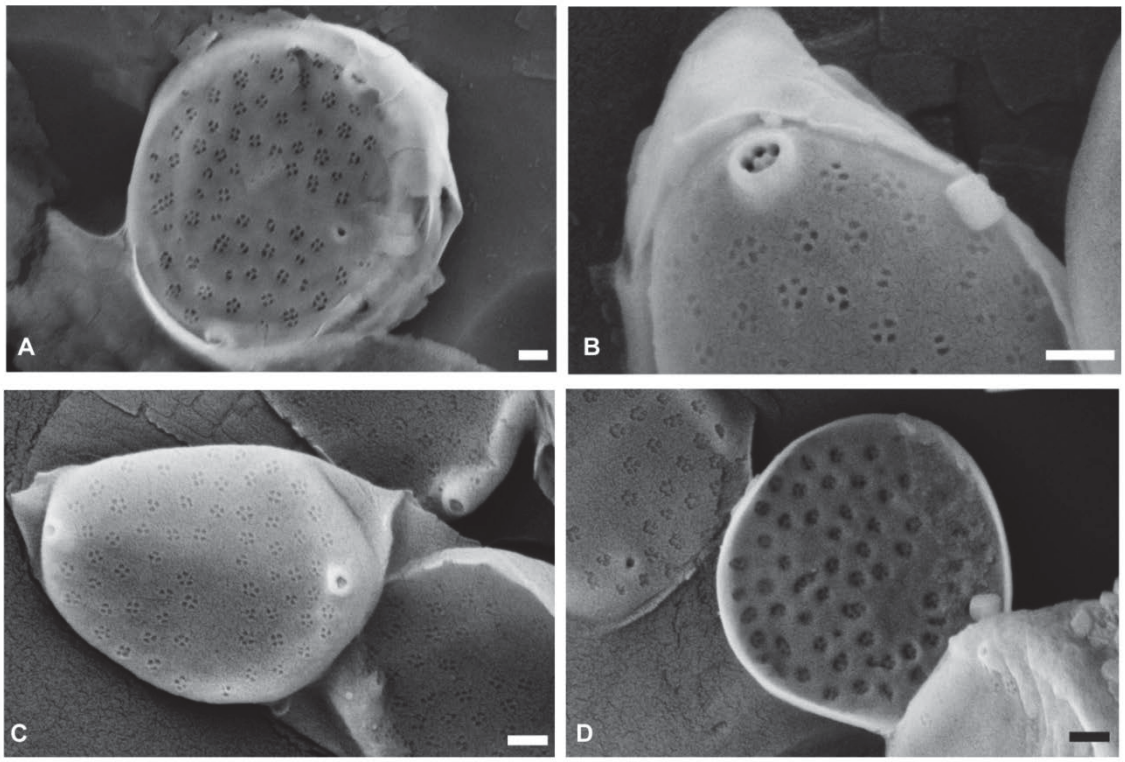


**Figure 3. Scanning electron microscopy (SEM) images of frustules of the diatom *Minutocellus polymorphus* found in cultures isolated from *Pararotalia calcariformata*. Scale bars represent 200 nm.**

To characterize the photosynthetic functioning of the discovered diatom endosymbiont, we measured the photosynthetic activity of the symbionts inside the host by Pulse Amplitude Modulated Fluorometry (PAM) (Fig. 4). To achieves this we conducted Rapid Light Curves (RLCs) after the protocol of Ralph and Gademann (2005), allowing us to assess the response of PS II (Photosystem II) to elevated light levels. The measurements on freshly collected specimens yielded an RLC (Fig. 4A), most similar to intermediate-light adapted *Amphistegina* (Nobes et al. 2008; Ziegler and Uthicke 2011) but we observed PSII photoinhibition (light adapted yields, Y(II)=0) at 166 µmol photons $m^{-2}$ $s^{-1}$. The RLC of *P. calcariformata* thus reveals an unusual sensitivity to high irradiance among the foraminifera, which we attribute to the combination of the different nature of the symbiont and the mid-latitude setting of the locality. Alternatively, the observed lack of fluorescence at higher irradiance levels

could be an artefact due to the behavior of the microalgae in the shell. On the other hand, the $ETR_{max}$ (maximum height of the curve) and the $E_k$ (minimum saturating irradiance level) of the curve fall exactly within the range of values determiend for other diatom-bearing foraminifera by Nobes et al. (2008) and Ziegler and Uthicke (2011).

The observed sensitivity to in-situ light intensities in *P. calcariformata* are in line with the observations by Nobes et al. (2008), who reported reduced $F_v$:$F_m$ and growth in *Calcarina* sp. cultured in high and mid-light treatments. This can be explained by the fact that foraminifera are motile and live in microhabitats that allow them to shelter their shell from excessive light. Because of the apparent light sensitivity of *P. calcariformata* the irradiance level in cultures and subsequent experiments was kept below 30 µmol photons $m^{-2}$ $s^{-1}$ PAR.

Next, PAM fluorometry was used to confirm the activity and persistence of the photosymbiosis among specimens collected during different seasons and in populations kept in culture for up to five months by measuring the dark adapted yield (maximum quantum yield; $F_v$:$F_m$). Field populations measured within one week of collection exhibit average $F_v$:$F_m$ of 0.60 in collections from both 11/2012 and 04/2013 (Fig. 4), indicating a seasonally persistent fully functional photosymbiosis in the studied population. The values are comparable to those determined in previous work on other species of symbiont-bearing Foraminifera (Nobes et al. 2008; Schmidt et al. 2011; Ziegler and Uthicke 2011). After one month of culturing the $F_v$:$F_m$ of the population collected in 11/2012 decreased to an average of 0.55. After longer exposure to laboratory conditions, the average $F_v$:$F_m$ decreased further between 11/2012 -03/2013 to 0.42 (70% of the initial value) and between 04/2013 -06/2013 to 0.45 (76% of the initial value). In the foraminifera *Marginopora vertebralis*, $F_v$:$F_m$ between 0.15-0.38 were still considered to represent functional photosymbiosis of the dinoflagellates symbionts (Sinutok et al. 2013). Thus, despite the reduced $F_v$:$F_m$, the *Pararotalia* specimens remained photosynthetically active in culture for several months, indicating that the symbiosis is of persistent nature.

The observed reduction of $F_v$:$F_m$ with time might be a sign of a reaction of the symbiont or the holobiont to the culturing conditions. In comparison to fluctuating light intensities and daily light peaks in their natural habitat (Nobes et al. 2008) the cultured specimens were exposed to low and constant light levels. On the other

hand, lower $F_v$:$F_m$ values could indicate nutrient stress in the cultures. It has been shown that low-light adapted diatoms have higher cellular iron needs to keep photosynthetic iron-based redox proteins functioning (Sunda and Huntsman 1997). Since the cultures were based on artificial seawater without addition of nutrients, it is possible that the feeding of the foraminifera with microalgae was not sufficient to allow for optimal nutrition. In contrast to this hypothesis, nutrient-limited cultures of the diatom species *Thalassiosira pseudonana* exhibit a constant $F_v$:$F_m$ ratio of 0.65 under balanced growth conditions (Parkhill et al. 2001). Therefore, it remains unclear whether the decreased $F_v$:$F_m$ indicates nutrient or light stress in the cultures, or whether it reflects the physiological state of the symbionts.

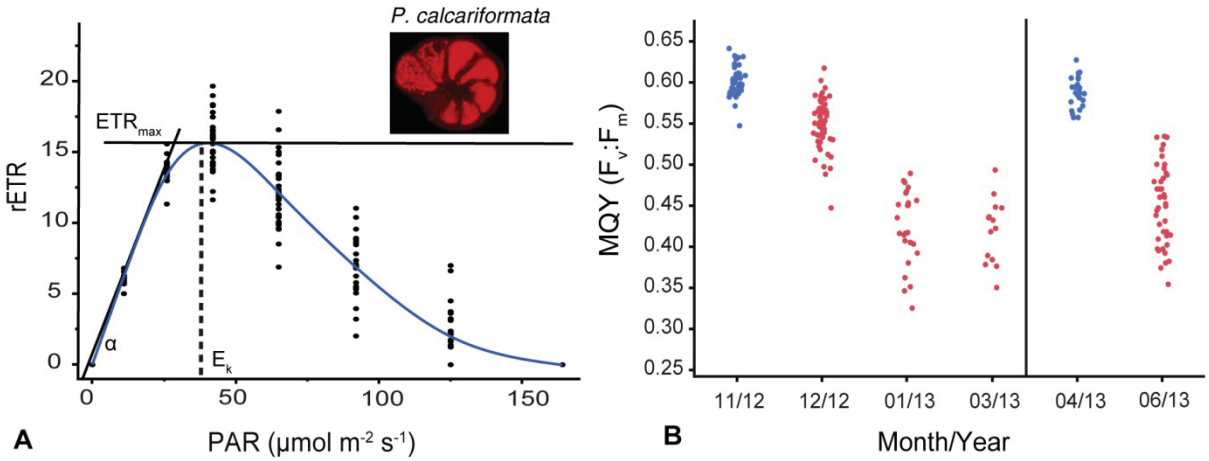


**Figure 4. Photochemistry of *Pararotalia calcariformata* symbionts. A) Rapid light curve showing relative electron transport rates (rETR) under different levels of photosynthetically active radiation (PAR) on low-light adapted specimens (n=30). The curve shows a cubic spline with a lambda of 0.05. (α) represents slope of the curve, (ETRmax) the maximum height of the curve and the (Ek) the minimum saturating irradiance level. Photograph shows a live specimen pictured under epifluorescence microscopy, showing photosynthetically active symbionts distributed throughout the shell. B) Maximum quantum yield ($F_v$:$F_m$) measured on two populations collected in 11/2012 and 04/2013 (n=14-48), blue dots represent measurements made one week after collection, red dots represent measurements of cultured specimens.**

### 3.4.3. Reproduction, growth and temperature sensitivity of asexual offspring

The extent of biological invasions and range expansions is limited by the ability of the species to establish a viable population at a new locality. Thus, next to the environmental tolerance of adult specimens, reproductive success of the expanding population is determined by the environmental suitability window of the reproduction event and of the survival and growth of juveniles, which can be a bottleneck for species survival under global change (Byrne 2012). Foraminifera are known to reproduce through a complex system of sexual (gametogenic) and asexual (multiple fission) cycles (e.g. Goldstein 1997; Hohenegger 2011). The length of the life cycle and the timing of reproduction is often poorly constrained, but continuous in situ monitoring over the full seasonal cycle shows highest abundances of *P. calcariformata* in the >0.63 µm size fraction in spring following natural temperature rise (Arieli et al. 2011), indicating that reproduction in this species in nature is likely to occur once a year in late spring.

We have observed asexual reproduction in populations in the laboratory cultures and were able to characterize the ontogeny under controlled laboratory conditions. After collections in 11/2012 and 04/2013, reproduction occurred at least once in each collection after 4-5 weeks of culturing, involving only a small number of the cultured specimens. The number of offspring per mother individual was relatively small: after reproduction in 05/2013, we observed app. 400 living three-chambered offspring and app. 20 dead potential parent individuals in the same culture. The offspring contained a minimum of three chambers when released from the parent and contained symbionts. The size and number of asexual offspring, as well as the presence of symbionts inherited from the parent are comparable to observations from laboratory experiments on many other species of symbiont-bearing foraminifera (Hohenegger 2011). Thus, the Levantine invasive population of *P. calcariformata* is able to reproduce under temperature, salinity and light conditions simulating the ambient setting in the Levant in autumn and spring. However, development under culturing conditions can only be an estimate compared to development under in-situ conditions because environmental factors, e.g. tides and lunar cycles were not simulated (Briguglio and Hohenegger 2014; Hohenegger et al. 2014).

To characterize the growth of the asexual offspring, six juveniles from the first reproductive event were kept in culture under constant conditions for 117 days. Three of the six individuals grew and developed new chambers filled with green-brownish cytoplasm (Fig. 5A,B). A twice weekly monitoring showed that the growth of the juveniles has two distinct phases. The first phase of rapid growth (lasting ~30 days) is followed by a phase of slow growth (Fig. 5A). All juveniles consisted of three chambers at the beginning of the experiment and measured up to 16-18 chambers at the end of the experiment. Until day 30, the chamber number increased from 4-(14-15). Thus, app. ten new chambers were formed in these three individuals in the first growth phase (Fig. 5B).To investigate the temperature sensitivity and development of asexual offspring we performed an experiment following the second reproductive event. The experimental setup consisted of three replicated plates per incubator exposing asexual offspring to 20°C, 28°C and 35°C over 48 days. The 20°C and 28°C cultures were set to simulate the natural range of temperatures in the eastern Mediterranean between spring and autumn (Herut et al. 2000; Arieli et al. 2011), with the 20°C culture representing the conditions at the time of collection. The 35°C treatment was chosen to establish the upper limit of offspring growth.

The results indicated that offspring mean growth was 0.86 % day$^{-1}$ in the 28°C treatment (Fig. 5C). Only in the 28°C treatment all observed specimens grew and the average growth curve of the population followed the same two-phase growth pattern as in the 2012 culture (Fig. 5D). The offspring kept at 20°C and 35°C survived the experiment, as seen by healthy coloration of the cytoplasm and cytoplasmic movement, but showed an inhibition of growth, indicating the lower limit of growth (Fig. 5D). This is in line with observations of benthic foraminifera by Alve and Goldstein (2002) where growth commenced only in individuals exposed to suitable environmental conditions. The proportion of individuals which showed positive growth under experimental conditions was 100% under 28°C, and was reduced to 64-65% in the 20°C and 35°C treatment. We statistically tested the effect of temperature on the individuals exhibiting positive growth. Temperature had a significant effect on growth rate on asexual offspring (One-Way ANOVA, $F=24.60$, $df=2, 34$, $p=0.0001$). The Tukey-Kramer post-hoc test revealed significant differences between the 28°C, the 20°C and 35°C treatments, but not between the latter two. Growth rates observed at 28°C in this study are comparable to those of sexually produced offspring of the benthic foraminifera *Planograbratella opercularis* (0.4-0.8% growth per day between 15-20°C) inhabiting a similar coastal environment on coralline algae in Japan (Tsuchiya et al. 2014). The observed inhibition of growth in the 35°C treatment, is consistent with an upper thermal limit for other species of symbiont-bearing foraminifera, where reduced growth, increased mortality and symbiont bleaching are observed at temperatures >31°C (Schmidt et al. 2011; Uthicke et al. 2012).

In contrast, the lack of offspring growth at 20°C was unexpected, considering that this was the ambient temperature at the time of collection and the reproduction in the laboratory occurred at that temperature. Our results indicate that although reproduction may occur at 20°C, the offspring needs temperatures >20°C in the subsequent weeks in order to initiate the rapid growth phase. Thus, the observation of reproduction at 20°C may be consistent with the lower temperature limit for offspring growth between 20°C and 24°C, provided the reproduction is aligned with the onset of the spring warming. This is consistent with the observation of maximum abundance in June and July in the >0.63 µm size fraction (Arieli et al. 2011). If the elevated abundance reflects reproductive events and the natural population follows the same growth pattern as seen in laboratory, then the reproductive in nature must

have occurred at least one month before the observed abundance maximum, i.e. in May or June.

If temperatures higher than 20°C are required for offspring growth in *P. calcariformata*, then the seasonal time window for reproduction in this species is longer in the Levant than in other areas of the Mediterranean (Shaltout and Omstedt 2014). It is possible that reproductive processes in *P. calcariformata* are incompatible with a narrower suitability window for reproduction shifted towards the summer in other areas of the Mediterranean. At present, between the Levant (e.g., Haifa) and the Ionian Sea (e.g. San Stefano, Corfu) a 20°C sea surface temperature threshold is shifted by three weeks from early May to early June and the total length of the >20°C thermal window is shorter by two months (IOLR 2010). This mechanism would provide a possible explanation for the restricted Levantine occurrence of the invasive species, as well as for its apparently recent invasion. The Levantine basin is known to have already experienced significant winter warming in the past decades and is predicted to increase its yearly mean SST by 0.5-2.3°C by the end of the century (Shaltout and Omstedt 2014; Sisma-Ventura et al. 2014).

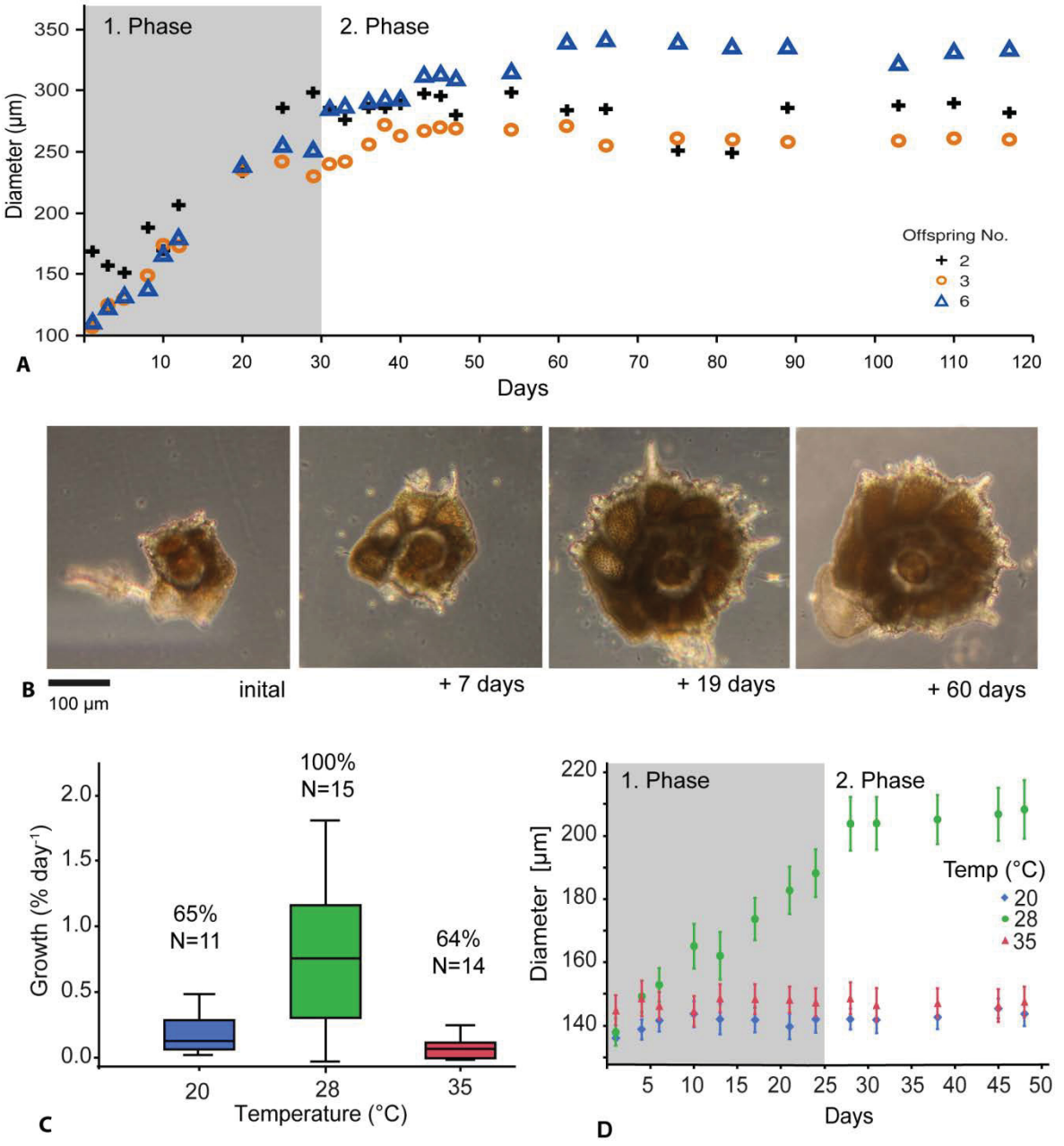


**Figure 5. Ontogenetic development of *P. calcariformata*. A) Shell diameter (µm) of three asexual offspring under culturing conditions showing a first rapid and second slower growth phase. B) Light microscopic images of Individual Nr. 2 from A) at different ontogenetic stages during culturing. C) Growth rates (% shell diameter increase day$^{-1}$) of asexual offspring in temperature sensitivity experiment. Percentage values indicate the number of individuals, which showed positive growth. D) Mean diameter (µm) of all asexual offspring over the course of the temperature sensitivity experiment. Error bars represent 1 SE.**

### 3.4.4. Modelling the present and future distribution of the species

If the limited distribution of *P. calcariformata* and its recent appearance in the Levant reflect unexpectedly low tolerance to cold temperatures, then it should be possible to express all the known occurrences of the species as a function of the local yearly temperature cycle. To explore this hypothesis we combined published occurrence records of the species with own sampling in the Mediterranean (Fig. 6) and used the data to calibrate a species distribution model linked to environmental variables at the observed sites. The variables we considered include solar irradiance and turbidity, which are relevant to the symbiont photosynthesis and yearly minimum temperature, representing potential limiting factors for the holobiont. The values that were used to calibrate the model represent multi-year averages for a time period after the first record of the invasive species (1997-2009 for irradiance and 2002-2009 for temperature and turbidity). The model setup and data source are as used in previous studies (Weinmann et al. 2013b).

The resulting species distribution model (SDM) (Fig. 6) indicates the highest habitat suitability in the Levant, along the coast of Israel and Lebanon. Moderately suitable habitat, representing “typical” conditions for a species, continues along the coast of Syria to southernmost Turkey, with its front corresponding exactly with the localities where the invasive species has been most recently reported by Meriç et al. (2013). The model also indicates the existence of suitable habitats along the Egyptian shelf, where the species has not been discovered so far (Samir et al. 2003; Elshanawany et al. 2011). However, we note that the most suitable habitat in this region is inferred to exist offshore, being reflected already in the three variables driving the model. Anthropogenic contribution of nutrients from agriculture and sewage has replaced the nutrient stimulating effect of the Nile discharge after closure of the Assuam damn in 1965 (Nixon 2003; Oczkowski and Nixon 2008), keeping conditions still sub-optimal for nutrient-sensitive organisms. Along the Levantine coast, *P. calcariformata* appears to preferentially inhabit the shallowest sublittoral environment, above the depth of 20m, indicating that this species needs well-lit, oligotrophic conditions. We note that similar to other benthic symbiont-bearing foraminifera, Pararotalia might therefore be a good indicator species for the FORAM Index as a proxy for ecosystem health in the Mediterranean, as shown for *Amphistegina lobifera* in the Aegean Sea (Koukousioura et al. 2011). In the studied

localities, *P. calcariformata* lives in association within turf algae or calcareous algae that inhabit rocky substrate in the sublittoral zone on abrasion platforms and beach rocks. As the Nile delta provides habitat lacking these conditions and because of the elevated nutrient load (Nixon 2003), it is not surprising that it may function as a natural barrier to a south-easterly coastal dispersal of the species.

Other species of *Pararotalia* have been reported to disperse with surface ocean currents attached to gastropod larvae (Cedhagen and Middelfart 1998). Whether or not attached to gastropod larvae, macro algae or transported as propagules (Alve and Goldstein 2002; Alve and Goldstein 2003), the dispersal of *P. calcariformata* would lead to passive northward transport along the Levantine coast following the persistent surface currents in the Levant (Stamou and Kamizoulis 2009; UNEP/MAP 2012). These currents move directly along the Israeli shelf and could facilitate the transport of plant material or larvae carrying attached *Pararotalia* towards the northern Levant, as it has been suggested for other benthic foraminifera (Langer 2008). This would mean that the main direction of current transport of the Levantine *Pararotalia* is further to the north, along the coast of Turkey and into the Aegean Sea. Here, the SDM indicates poor habitat suitability, due to colder temperatures (Fig. 6). Indeed, the species does not appear to have invaded the Aegean Sea yet (Koukousioura et al. 2011; Frontalini et al. 2014). A recent detailed investigation of foraminifera diversity in the Saros Bay, a region with abundant suitable habitats for *P. calcariformata*, has not identified this species among the 115 species recorded (Frontalini et al. 2014).

To investigate whether the species range given by the SDM derived from its present-day distribution is likely to expand further, we have evaluated habitat suitability under a realistic (intermediate scenario A1B) global change projection for the year 2100 (Jueterbock et al. 2013). Predicted habitat suitability increases drastically along the coast of Turkey, suggesting a high probability of invasion in this region within the next decades. In addition, the results imply a large potential for an ongoing expansion of the species into the Aegean Sea and the Greek and Libyan coasts of the Ionian Sea.

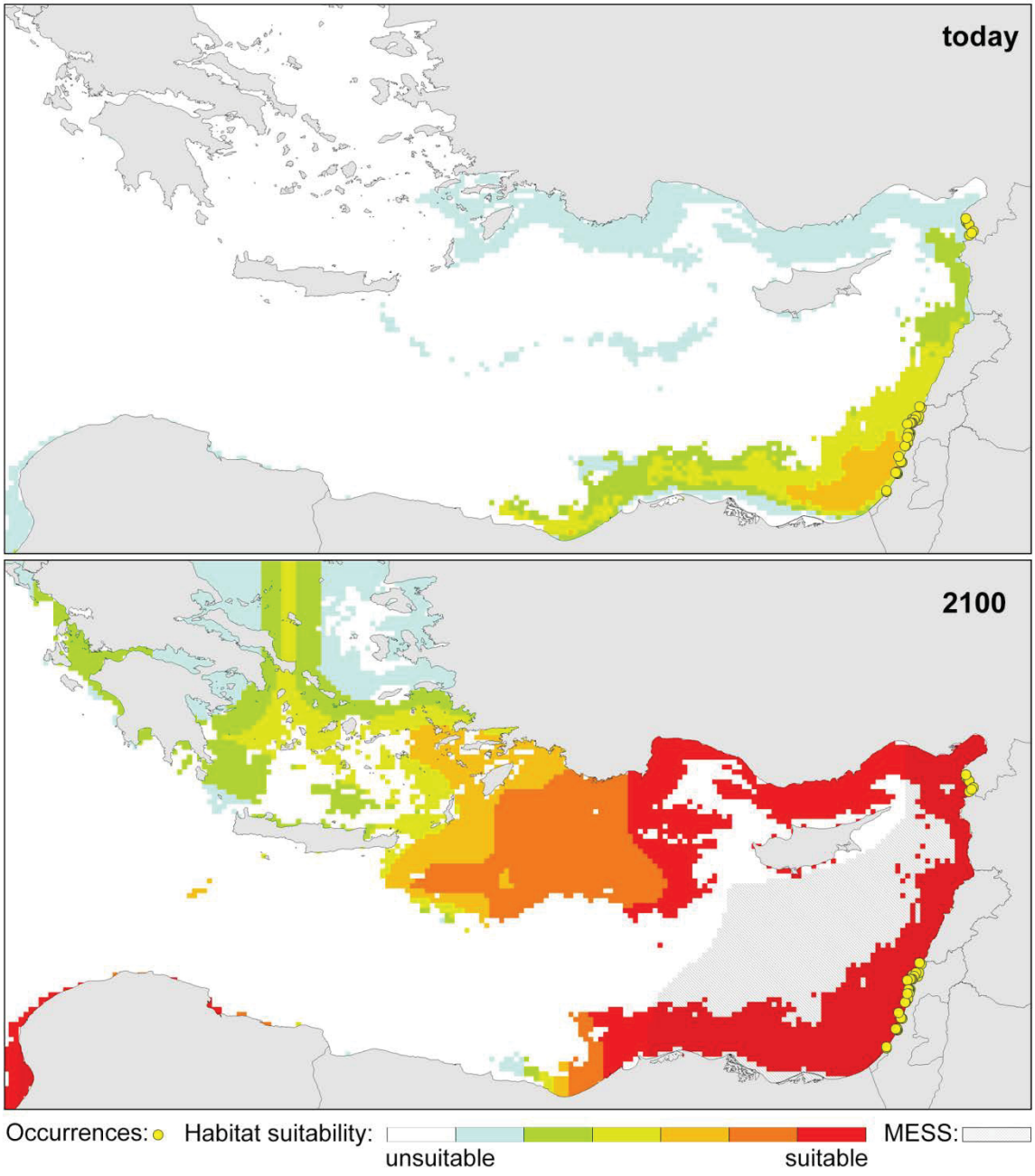


**Figure 6. Occurrence records of *P. calcariformata* overlain with a habitat suitability values from a species distribution model based on yearly minimum temperature, mean light attenuation and mean PAR (photosynthetically active radiation) for the present day and under projected future conditions (intermediate global change scenario AB1). MESS indicates the results of the multivariate similarity surface analysis indicated by grey stripes.**

## 3.5. Conclusions

A taxonomic revision of the most recently discovered invasive species of symbiont-bearing foraminifera of the genus *Pararotalia* in the eastern Mediterranean identifies the invader as *P. calcariformata* McCulloch 1977. Based on phylogenetic and taxonomic evidence, the invasive population appears to have originated from within the Pacific radiation of the genus. Pulse amplitude modulated fluorometry measurements indicate that the species is engaged in permanent symbiosis with photosynthesizing microalgae. Combined culturing and genotyping approach allowed us to identify these algae as a consortium of small diatoms, including the newly described symbiont *Minutocellus polymorphus*.

*Pararotalia calcariformata* has been observed to asexually reproduce and grow in manipulative experiments, revealing an initial 30-day period of rapid growth followed by a slower growth phase. Reproduction occurred at 20°C, but normal offspring growth was only observed at 24°C and 28°C, indicating that a successful establishment of populations of the species may be limited by the length of the thermally defined reproductive window in their habitat. To test this hypothesis we derived a SDM based on present day occurrence of the species and show that together with turbidity and irradiance, yearly minimum temperature alone is sufficient to reproduce the observed species range with remarkable fidelity. This model indicates that the species is likely to continue expanding northwards and westwards under realistic global change scenarios and is likely to reach the Ionian Sea by 2100.

Collectively, the evidence indicates that the symbiont-bearing foraminifera *P. calcariformata* is likely a Lessepsian migrant whose invasion into the Mediterranean has been facilitated by the recent warming in the Levant (Shaltout and Omstedt 2014). In this way, the case of *P. calcariformata* adds to mounting evidence for ongoing and dramatic changes in the structure of eastern Mediterranean ecosystems (e.g. Hiddink et al. 2012; Edelist et al. 2013). Its invasion reflects multiple aspects of human-mediated dispersal of marine species – environmental change due to global warming and removal of physical barriers. It shows that in the absence of limitation to dispersal, the invasion rate is almost unlimited with ongoing range expansion (Meriç et al. 2013) observable on sub-decadal time scale.

## 3.6. Acknowledgments

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## 3.8. Supporting Information

**S1. Species description of *Pararotalia calcariformata* in the Mediterranean, with a documentation of shell morphology of late ontogentic stages (Plate 1) and juveniles (early ontogenetic stages, Plate 2).**

**Taxonomic description and documentation of morphology of the invasive Mediterranean *Pararotalia calcariformata***

Superfamily ROTALIOIDEA Ehrenberg, 1839

Family ROTALIIDAE Ehrenberg, 1839

Subfamily PARAROTALIINAE Reiss, 1963

Genus *Pararotalia* Le Calvez, 1949

*Pararotalia calcariformata* McCulloch, 1977

Not *Pararotalia spinigera* (Le Calvez, 1949), emend. by Loeblich and Tappan (1957), Plate 4, Figs 1-3, [middle Eocene, Lutetian, Calcarie Grossier, France]

Not *Pararotalia spinigera* (Le Calvez, 1949), Hottinger et al. (1991) Plate 1 Figs. 2, 5-8 [middle Eocene, Lutetian, Grignon, Paris Basin, France]

*Pararotalia* (?) *calcariformata* McCulloch, 1977, McCulloch (1977), Plate 177, Figs 10-11, [Recent, type locality Colombo Bay, shallow waters, Station 616, off West Nole Island, Australia, shallow waters]

*Pararotalia calcariformata* McCulloch 1977, Loeblich and Tappan (1994), Plate 367, Figs. 10-13, [Hypotype, Southern Timor Sea, Figs 11-13 sample V-347 at 58.52 m [Recent, North of Bathurst Island, southeast Timor Sea, 1961 Sahul Shelf Cruise II Stranger)

*Pararotalia spinigera* Le Calvez 1949, Reinhardt et al. (1994), Plate 2, Figs 11-12. [Late Holocene, Recent, CT/Grab 3, 9 m, Caesarea, Israel]

*Eponides repandus* Fichtel et Moll, 1798, Yanko et al. (1994), Plate 2, Figs. 1-9 [Recent, Haifa Bay, Station 27, 12 meters]

*Pararotalia spinigera* (Le Calvez, 1949), Arieli et al. (2011), Plate 6, Figs. 9-12 [Recent, Hadera, Israel]

*Pararotalia* (?) *calcariformata* McCulloch, 1977, Meriç et al. (2013), Plate 1 Figs. 1-11, not 12 [Recent, Hatay, Turkey, 3-8 meters]

**Original species description:** "Test free, calcareous, auriculate, biconvex, trochospiral, periphery lobulated, craniate with usually one short spine per chamber, dorsally wall rather smooth semi-hyaline, finely perforate; dorsal side evolute showing less than three whorls of slightly inflated subrhomboidal chambers gradually increasing in size; sutures dorsally limbate darker hyaline flush to depressed curved bands. Ventral side involute with prominent umbilical, raised nodulous, hyaline to umbilical plug surrounded by nodulous umbilical flaps, usually seven chambers in the last formed whorl; keel shallow hyaline acute with most of the tapering peripheral chambers forming a single short angular hyaline spine extending out in posteriorly from anterior half of each chamber of last formed whorl; sutures ventrally rather broad limbate depressed radiate; aperture interiomarginal extraumbilical umbilical with a narrow depressed rim outlining a low arch beginning at the close to the periphery, no tooth plate visible due to excess shell on most specimens.

**Comments**: The Levantine *Pararotalia* examined by us (see Plate 1 and 2) reveals a significant morphological variability, which is accentuated by ontogeny. Juvenile forms (Plate 2) typically possess trapezoidal chambers and single pseudospines (occasionally two pseudospines per chamber) extending from most chambers of the last whorl, occasionally positioned near the septal face. Chambers of the last whorl in fully developed adults are usually petaloid and lack the peripheral pseudospines. The peripheral margins and keel of the umbilical side in both juveniles and adults are covered with numerous short pustules, found also in *P. spinigera* (Le Calvez 1949).

The studied specimens from Israel reveal two pore structures that have not been documented previously: micropores and larger circular to elliptical pore mounds on the surface of both sides of the test in juvenile and adult forms. The chamber wall of *Pararotalia sp.* is highly transparent, similar to other foraminifera known to host symbionts such as other Calcarinids. It has been suggested that such a wall structure can be seen as an adaptation to photosymbiosis (Leutenegger 1984). The range of variability in the examined populations include all morphologies figured by Meriç et al. (2013) .These authors note the lack of spines in their specimens, but we believe they do so in contrast to species of *Pararotalia* with more prominent spines, such as *P. stellata*. Not all specimens of the Levantine studied by us had spines (Plate 1, 1-3). Also, we note that a specimen pictured by Meriç et al. (2013) in their Figure 2, image 4a, seem to possess spines of a similar shape and extent as in the studied population from Israel (Plate 1, 4). Therefore, we conclude that the populations from Israel and Hatay, Turkey, are morphologically overlapping. Following Meriç et al. (2013) we conclude that the Levantine *Pararotalia* is morphologically distinct from the concept of *P. spinigera*. The latter species has a lobate periphery without keel, which is a distinct and persistent feature of the Levantine population (Plate 1, 2-4, 6). In addition, in the Levantine population the umbilical sutures are so deeply incised that the walls of adjacent chambers become partly disconnected, forming deep interlocular spaces (Plate 1, 1, 3, 4, 6). This character is indicated in the illustration of the type material of *P. calcariformata* (McCulloch 1977).

## Plate 1

### Late ontogenetic stages of *Pararotalia calcariformata*

**1.** Adult specimen with dextral coiling. **a-b** spiral views, note the slightly raised sutures, a rounded peripheral outline, the spinose peripheral keel, and the central thickening of the early whorls. **b**. Details showing densely perforated pore fields between imperforate septal sutures. **c-d** Umbilical (ventral) views, note the interlocular space between consecutive chambers, the presence of umbilical plug fused with the umbilical wall of the surrounding chambers, the thick marginal nodes on early chambers, and the hirsute peripheral surface with numerous pustules.

**2. a.** Spiral view of fully developed adult specimen (sinistral coiling), with nine chambers in the last whorl. **b-c** Lateral view of adult specimen showing interiomarginal aperture

**3.** Adult specimen with dextral coiling. **a-b** Spiral view with a frequent well-developed pore mounds. **c-d** Umbilical view showing umbilical plug, large nodes along the edges of the sutures and chamber surface with well-developed pore mounds. **e.** Lateral view showing interiomarginal aperture.

**4.** Adult specimen with dextral coiling. **a-b** Spiral views showing nodose ornamentation, peripheral spines on early chambers, and microperforate surface of the ultimate chamber. **c-d** Umbilical views showing details of a peripheral spine. **e** Lateral view showing interiomarginal aperture and keeled outline.

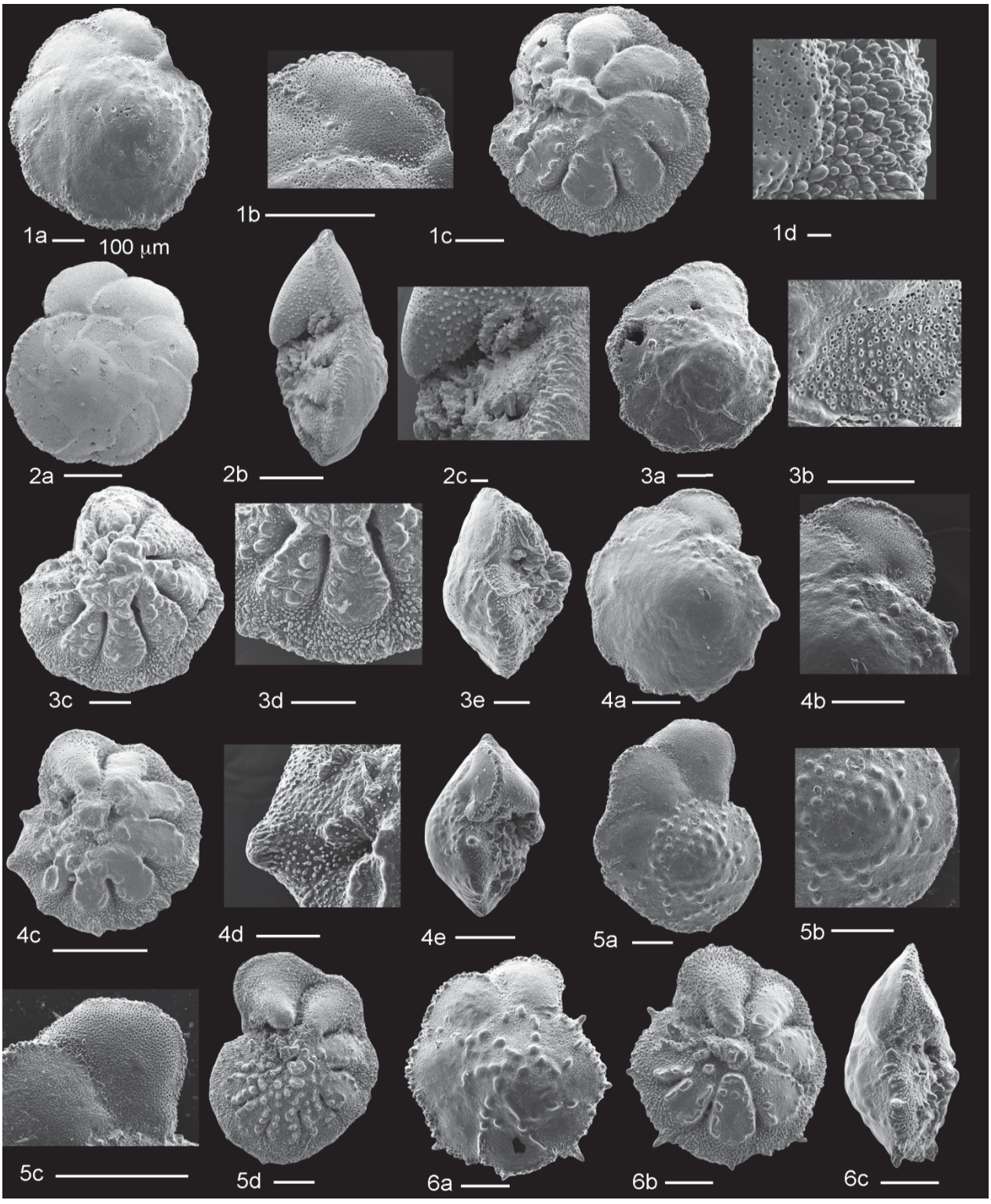
**5.** Adult specimen with sinistral coiling. **a-c** Spiral side, note the early nodose whorls and the microperforate surface of the ultimate chamber. **d** Umbilical view showing strong nodose ornamentation.

**6.** Adult dextral coiled specimen with peripheral spines. **a** Spiral view. **b** Umblical view.

**c** Lateral view.

Scale Bars: 100 μm

**Plate 1**


1a
100 μm
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2b
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3e
4a
4b
4c
4d
4e
5a
5b
5c
5d
6a
6b
6c

## Plate 2

## Early ontogenetic stages of *Pararotalia calcariformata*

**1.-3.** Earliest stage specimens with three and four chambers. **1** Spiral view of specimen with incipient peripheral pseudospines. **2** Spiral view of specimen with peripheral pseudospines. **3**. Umbilical view of specimens without peripheral pseudospines, showing incipient keel. Note the pustulate surface.

**4.** Juvenile specimen with peripheral pseudospine on each chamber. **a**. Spiral view.

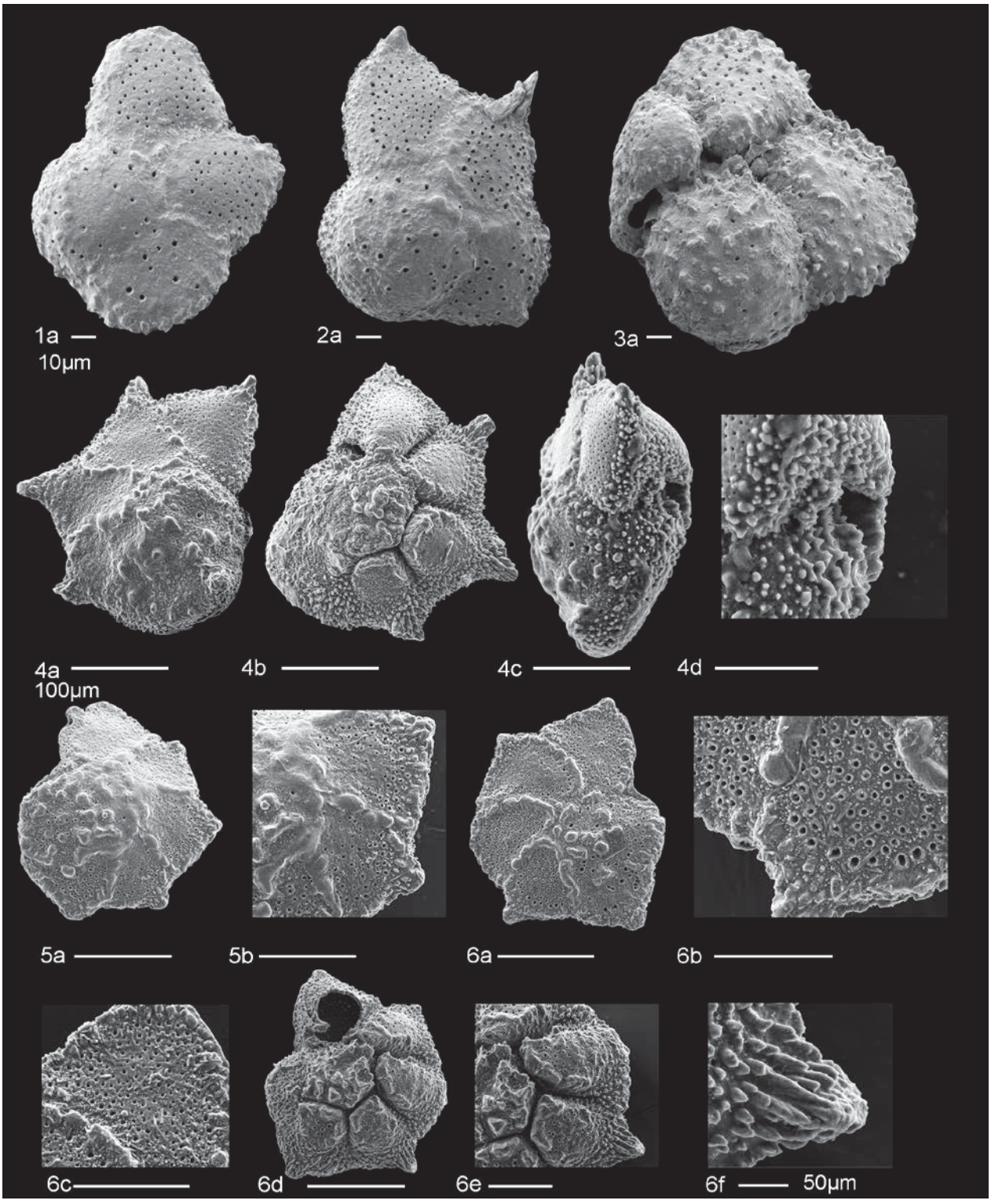
**b** Umbilical view, note the distinct trapezoidal shape of the chambers in the spiral and umbilical sides. **c** Lateral view. **d** Details of the interiomarginal aperture.

**5.** Juvenile specimen with peripheral pseudospine on each chamber. **a- b** Spiral views showing well-developed pore mounds and trapezoidal shaped chambers.

**6.** Juvenile specimen with peripheral pseudospine on each chamber. **a- c** Spiral views. **d-f** Umbilical views, note the relatively large pore mounds on the surface both sides of the test. **f** Details of the peripheral pseudospine.

Scale Bars: 1-3: 10μm, 4-6d: 100μm and 6e: 50μm

**Plate 2**



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# Chapter 4

# 4. Publication III: Extreme heat tolerance of a foraminifera–diatom photo-symbiosis

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## 4.1. Abstract

The loss of algal symbionts induced by thermal stress (bleaching) has been observed in many photo-symbiotic eukaryotes, such as corals, foraminifera and molluscs. In almost all documented cases, the bleaching threshold is close to the highest temperatures occurring at present in the open ocean (31-32°C). It appears that elevated temperatures universally compromise the stability of the eukaryotic photo-symbiotic relationship and that the physiological tolerance limit may be exceeded in many parts of the tropical oceans under realistic global change scenarios. Here we take advantage of the recent discovery of the occurrence of the diatom-bearing foraminifera *Pararotalia calcariformata* at a thermally polluted coastal site in Israel, where water temperatures seasonally reach 36°C. To test whether this thermal adaptation is universal in *P. calcariformata* we conducted manipulative experiments exposing this species together with *Amphistegina lobifera* from an unpolluted side in the eastern Mediterranean to elevated temperatures for up to three weeks. We measured the photosynthetic activity of the photo-symbionts and recorded survival and growth of the organisms. Reduced photosynthetic activity was recorded for *A. lobifera* above 32°C, but *P. calcariformata* showed a higher tolerance. Photochemical stress in *P. calcariformata* was first observed during exposure to 36°C after three weeks and chronic photoinhibition (but not mortality) first occurred at 42°C after one week. Survivorship was high in all treatments, and growth was observed at 35 and 36°C. It appears that *P. calcariformata* exhibits the most thermally tolerant photosymbiosis observed to date in a eukaryote-eukaryote system. The thermal tolerance is present in a natural environment where the thermal threshold is never realized. Observations imply that marine eukaryote-eukaryote photosymbiosis is more resistant to temperature than expected and this life strategy is likely to persist in a warmer world.

## 4.2. Introduction

To increase our understanding of the impacts of global climate change on marine ecosystems, we need to gain more specific knowledge on the thermal limits and adaptation potential of a diverse range of marine species. Specifically, it is essential to characterize the physiological and evolutionary traits that determine if a species is highly climate change vulnerable or is likely able to adapt (Foden et al. 2013). Species have been shown to respond negative but variable to global change stress (Kroeker et al. 2010). This might be due to different physiological adaptive capacities (Füssel and Klein 2006) and different eco-physiological processes which limit the fitness and survival of sensitive organisms (Pörtner 2002). Experimental work has shown that under high temperatures, excessive oxygen demand in the cells leads to falling oxygen levels in the cell fluids, reducing the metabolic capacity of organisms (Pörtner 2002; Pörtner 2010). In addition, higher temperature causes higher metabolic rates, which stimulate increased production of reactive oxygen species (ROS) (Sohal and Weindruch 1996). These are damaging to protein function and membrane structure and are thus reducing the fitness of the exposed organism (Sohal and Orr 2012). In eukaryotes hosting an endosymbiont, the damage not only occurs in the host but also in the endosymbiotic partner. In corals, it has been shown that the combined physiology of the holobiont is so interlinked, that either stress in the symbiont or the host, determines the holobiont's thermal tolerance (Bhagooli and Hidaka 2003; Goulet et al. 2005; Visram and Douglas 2007). If stressful conditions increase over a threshold and the advantage of the symbiotic relationship is lost, the zooxanthellae are expelled, a process which is known as bleaching (Brown 1997).

Under natural conditions, photosymbiosis provides an advantage in oligotrophic conditions (Brasier 1995). It allows the host to feed on the algae-derived 'photosynthates' and, the host in return protects the symbionts in the nutrient enriched cytoplasm (Hallock 1981; Lee and Hallock 1987). However, if the thermal tolerance of the host or symbionts is exceeded, bleaching can lead to mortality of entire reefs (Berkelmans and Oliver 1999; Wooldridge 2009). Because bleaching in the field can be associated with more than one factor, bleaching thresholds should not be confused with thermal thresholds, as the latter are only testable under controlled environmental conditions. In corals, bleaching stress has been associated with symbiont diversity. Clade C *Symbiodinium* has been shown to be more heat-

resistant than *Symbiodinium* clade D (Rowan 2004), but different bleaching susceptibilities have been found even within clade C *Symbiodinium* (Abrego et al. 2008; Sampayo et al. 2008). Despite the existence of variable thermal tolerance, most marine photo-symbiotic systems involving eukaryotes do not appear to tolerate temperatures that are substantially exceeding the upper thermal limit of tropical ocean water and bleach after exposure to 30-34°C (Fitt et al. 2001; Coles and Riegl 2013; Fine et al. 2013). The most heat-tolerant coral has been shown to be the massive coral *Porites solida,* tolerating temperature of up to 34°C for 12 h (Strychar et al. 2004; Strychar and Sammarco 2009). The most resistant reef to bleaching are located in the Arabian Gulf, where corals experience naturally the greatest temperature fluctuations worldwide, and have been shown to survive 36°C for a short period of time without signs of stress in the 1980es (Coles 1988; Coles and Riegl 2013) Indeed, under normal marine conditions of the late Cenozoic (Zhang et al. 2014) organisms adapted to higher thermal tolerance would have had no adaptive advantage and an evolution of higher tolerance would appear to be meaningless from an evolutionary perspective.

In benthic foraminifera, upper limits of foram-algae thermal tolerance, have been documented as tissue damage, reduced growth rates, increased mortality, reduced photosynthetic rates and bleaching onsetting at 31°C(Hallock and Talge 1993; Schmidt et al. 2011; Uthicke et al. 2011). In comparison to corals, benthic foraminifera establish an endosymbiosis with different algae types such as diatoms, dinoflagellates, chrysophytes, rhodophytes, and also cyanobacteria (Pawlowski et al. 2001; Lee 2006). On the host level, their response to elevated temperatures has been shown to be species-specific. For example, the diatom bearing *Calcarina mayorii* was not affected by temperatures up to 31°C but *Calcarina hispida* bleached in experimental studies at the same temperature (Schmidt et al. 2011). Thus, like corals and other photo-symbiotic marine organisms, tropical foraminifera appear to be vulnerable to warming levels predicted for the next century by realistic global change scenarios (Fitt et al. 2001).

In benthic foraminifera, there appears to be one exception to this general pattern in upper thermal thresholds. In 2011, Arieli et al. (2011) reported the occurrence of the diatom-bearing species *Pararotalia calcariformata* in a thermally polluted site on the Mediterranean coast of Israel, where this species seems to

tolerate long term exposure to 36°C for several month (Arieli et al. 2011). This is well above, the bleaching threshold of most studied marine eukaryotic photo-symbiotic systems, such as most corals species (Fitt et al. 2001; Fitt et al. 2009), except extremely thermally tolerant corals in the Gulf of Aden, which can tolerate up to 36°C without signs of stress (Coles and Riegl 2013), Indo-pacific foraminifera (Schmidt et al. 2011; Uthicke et al. 2012; van Dam et al. 2012) and mollusks (Leggat et al. 2003). However, it has not yet been shown whether the photosymbiosis in this benthic foraminifer, *P. calcariformata* also takes place at these temperatures and it remains unclear whether the resistance evolved within the unique environment of the thermal plume or whether it is a universal property of the species.

To test these assumptions, we collected a population of this species from a natural unpolluted location, 18 km north of the heat polluted site and exposed it to elevated temperatures. We exposed specimens of the summer and winter populations inside replicated sea water aquaria to temperatures up to 36°C for up to three weeks. As a consequence of its heat resistance we then performed an extreme test experiment, exposing to up to 42°C for three weeks in aquaria inside incubators. We monitored weekly photosynthetic activity of the symbionts using Pulse Amplitude Modulated (PAM) Fluorometry and recorded survivorship and growth, as an indicator for holobiont fitness. To demonstrate the representativeness of our laboratory conditions, the first two experiments included populations of the *A. lobifera* from the same locality. The diatom-bearing genus *Amphistegina* has a known thermal tolerance and species of this genus have been previously shown to bleach at 32°C (Talge and Hallock 2003; Schmidt et al. 2011).

## 4.3. Methods

### 4.3.1. Sample collection

Living specimens of *Pararotalia calcariformata* and *Amphistegina lobifera* were collected in the eastern Mediterranean Levantine basin at Nachsholim (32° 37.386 N, 34°55.169 E). Samples were collected by sampling filamentous coralline algae e.g. *Jania sp*. from the site, a shallow coastal high-energy habitat, by snorkeling at 0.5-2 m water depth on three field campaigns 1/11/2012, 08/04/2013 and 23/10/2013. Samples were transported in large plastic bottles filled with algae and sediment to the

laboratory, where the algae and sediment were rinsed with sea water and specimens were picked from the concentrated sediment. Several specimens (up to 50) have been put inside screw capped plastic jars (Volume 100 mL) together with some algae and sediment material. The jars were express-shipped inside an insulation container to Germany, experiencing minimal temperature fluctuations. During two shipments on 22/10/13 and 24/10/13 temperature was logged every 30 min (Hobo, Temperature logger) showing mean temperatures of 22.2 -23.2°C, SD 2°C (duration of shipments 24- 36 h). The specimens were cultured inside plastic containers at in-situ water temperature of the collection (23-24°C in November, 20-21°C in April) under a diurnal light cycle 12 h/12 h (light conditions of <30 µmol $m^2$ $s^2$) in incubation chambers at ambient salinity (38.5-40.0 ‰). Half of the sea water was replaced every week by freshly made seawater (Tropic Marine Sea Salt, Germany).

### 4.3.2. Experimental design and sea water parameters

A replicated design of 12 aquaria (working Volume 18 L) was installed on a laboratory bench top for conducting the summer (SuPE) and winter exposure (WiPE) experiments exposing the two species to 5 consecutive temperatures. In each aquaria temperature was manipulated separately using heating rods controlled by an aquaria computer system (AT control, Aquamedic, Germany), which are automatically switched on or off when temperatures in the aquaria would fall below or be elevated from the set deviance of +/- 0.1°C from the inserted temperatures. Seawater temperatures were recorded every 10 min by the software and saved. As the 20°C treatment during the winter population experiment (WiPE) was below the ambient temperatures of the room, two aquaria were externally cooled by an external water cooler (Titan 150, Aquamedic). For this the aquaria were by put inside two large plastic boxes (working volume XL) and were connected to a pump (Ocean Runner 1200, Aquamedic) and the water cooler, which constantly circulated water around the aquaria cooled to below room temperature, which was then precisely heated by heating rods of the Aquamedic system, inside the aquaria, to the desired 20°C. Temperatures during the SuPE were between 24-35°C (duration: 2 weeks) and during the WiPE between 20-36°C (duration: 3 weeks) (Fig. 2). We choose to slightly adjust the temperatures and the duration of the experiments focusing during the WiPE on the warmer end of the temperature spectrum, taking into account results of SuPE suggesting a higher stability of thermal thresholds in *P. calcariformata*. Inside

the aquaria, water movements were mimicked by using strong flow pumps (NanoProp 5000, Aquamedic). Manual temperature measurements were conducted daily using a handheld thermometer and salinity meter (WTW, Germany) and showed that there were minimal daily variations from the set temperatures (+/-0.5°C). The aquaria were lighted from top on a 12h day /12 h night cycle by daylight fluorescent bulbs (50:50 actinic 420nm/ 10 K trichromatic). Photosynthetic active radiation (PAR) varied inside aquaria and was recorded at the beginning and at the end of the experiments and varied between 35-40 µmol $m^2$ $s^2$ (Apogee MQ-200, USA).

For the extreme temperature experiment (ExTE), temperature controlled incubation chambers (Pol-Eko-Aparatura, Model ST2+/ST2+, Poland) were used, as temperatures were aimed to be set to four temperatures 24-42°C (Fig. 3) for the duration of 3 weeks. The upper thermal temperature of 42° is not recommended for the Aquamedic setup, therefore we used the incubation chambers for temperature control. Each incubation chamber contained two plastic mini-aquaria (working volume 2 L) standing on the same level to be illuminated with constant light conditions (diurnal light cycle 12h /12 h, 19-26 µmol $m^2$ $s^2$, white-fluorescent light bulbs). The aquaria were ventilated by bubbling air by small hand-held air pumps into the seawater (duration for 30 min each). For automatic temperature monitoring including diurnal variations, one aquaria per incubator contained a temperature logger (Hobo, USA). Logger data was excluded from the data set when organisms were taken outside for measurements. Temperatures varied by app. +/- 1.3°C from the set temperatures. Exact Logger means per treatment were (N=931, measurements ever 30 min) were for the 24°C treatment, 24.0°C (SD 0.42), for the 30°C treatment 29.9°C (SD 0.44), for the 36°C treatment 36.3°C (SD 0.53), for the 42°C treatment 42.5 (SD 0.65). Those where used to name the treatments accordingly in Fig. 3 and 5. Manual temperature measurements inside the aquaria were made daily using a handheld meter (WTW, Germany) for monitoring the incubator temperatures and were slightly below the logger temperatures, as they do not reflect higher night temperatures because lights where off: incubator 1 (24°C treatment)= 23.6°C (SD 0.19), 2 (30°C treatment) =29.4°C (SD 0.27), incubator 3 (36°C treatment)=35.4°C (SD 0.42), incubator 4 (42°C treatment)=41.2°C (SD 0.55). Diurnal differences (day/night) were only monitored by the loggers and were for the 24°C treatment 23.6°C/24.3°C (SD 0.28, 0.28); for the 30°C treatment 29.6°C/30.2°C

(SD 0.33/0.29); for the 36°C treatment 35.9°C/36.7°C (SD 0.41, 0.25); and for the 42°C treatment 42.0°C/43.0 (SD 0.53/0.32).

Sea water was made in bulk using artificial sea salt (Tropic Marin® Sea Salt, Germany) at the beginning of the experiments. Salinity measurements were made daily with the same instrument as temperature was checked (WTW, Germany) and was adjusted to stay in the range between 38.5- 40.2 ppm in all experiments, by adding deionized water when needed. These levels are generally observed in the Levantine Basin and represent in-situ conditions for the experimental species (Herut et al. 2000). The pH levels of the seawater were monitored weekly and stayed > 8.1 pH units.

At the beginning of the experiment and after weekly measurements, foraminifera were fed with marine microalgae (frozen, autoclaved) by adding 15 µL of food mixture to the glass jars. Food mixture consisted of *Nannochloropsis* algae concentrate (12 x $10^9$ cells/mL, BlueBioTech GmbH, Germany) which was diluted with sea water (30µL concentrate: 200 mL seawater) and autoclaved.

The housings of the foraminifera were prepared to provide wall surface to allow specimen to climb up walls using their pseudopods, and at the same time, provide enough protection for specimens inside the water flow of the aquaria. Previous studies also quantitatively described the motility of the specimens (Schmidt et al. 2011), which is especially interesting in Amphistignidae, because they are very motile, but as *P. calcariformata* was observed to be generally be much less motile and is generally very small (0.3 µm), we did not examine this parameter here. 5-6 specimens were kept inside a snap-cap top vial inside the aquaria and covered by a flow-through top which allowed passive water exchange inside the chambers. Therefore, in the top of a standard snap-cap top vial (Volume 15 mL, Wheaton, UK) a large hole was cut which hold in place a small piece of plankton mesh net underneath (*P. calcariformata* mesh size of 100 µm and *A. lobifera* 300 µm). Six snap-cap vials were put inside each aquarium on the bottom of a standard 6 well-plate to ensure stability, containing either *A. lobifera* or *P. calcariformata* in a vial. After weekly measurements the arrangement of the vials inside the aquaria was randomized to reduce bias due to different positions inside the aquaria, after mesh has been cleaned with water from algae to ensure same light conditions throughout the experiment.

### 4.3.3. Photochemistry measurements

To carry out photochemistry measurements on PS II, a chlorophyll fluorometer IMAGING-PAM *M-Series* Fluorometer (WALZ GmbH, Germany) was used. It was equipped with MAXI-Head,1/2'' CCD camera and zoom objective (F1.0/f=8-48 mm). Foraminifera of each snap-cap vial were transferred by sucking with 10 mL pipette into petri dishes containing the treatment water and dark-adapted 10-20 minutes before measuring dark-adapted yield (=maximum quantum yield, MQY, $F_v$:$F_m$). Light-adapted yield (effective quantum yield, EQY, Y(II)) was measured under light adaptation similar to experimental light levels, supplied by LED lights installed in the MAXI-Head. Measurements were conducted once per week. We elevated the Petri dishes closer to the zoom objective on a 1.5 cm-high stand for *P. calcariformata* (size 0.3-0.4 µm) and used the Leaf Holder IMAG-MIN/BK to allow best possible imaging for all specimens. Light levels of the LED light units were measured with PAR Light Meter (Apogee, USA) and were 25 µmol photos $m^2$ $s^2$, similar conditions as in the aquaria and incubator setup. Other procedure on data processing is given in previous work on foraminifera using the IPAM (Schmidt et al. 2011; Uthicke et al. 2012; Vogel and Uthicke 2012; Schmidt et al. 2014).

### 4.3.4. Survivorship & Growth measurements

Visual assessment of the foraminifera to determine survival rate was carried out when high resolution photographs have been made at the onset, and at the end of the experiments. Foraminifera were photographed at a resolution of 5184 x 3456 Pixel using a Canon SLR camera mounted on a Zeiss stereomicroscope. As individual experimental units (snap-cap vials) containing the foraminifer were not individually tracked during the SuPE and ExTE, mean growth rates per aquaria where calculated from measuring the surface area from initial versus final images of the specimens after the formula given in Schmidt et al. (2011). Aquaria (SuPE and ExTE) or wells (WiPE) were excluded from the data set where more specimens than initially inserted where recovered, to eliminate bias because of individuals which might have moved or reproduced. Reproduction only occurred in a few specimen during each experiment (SuPE under 32°C, and in WiPE at 34°C, and at ExTE under 36 °C), resulting in negative growth rates of the aquaria, which have been removed from the data set. Missing and dead specimens have been identified by sorting the

diameters by size or by individual features and removed from the growth data set to eliminate bias. Slightly negative growth rates in the 36°C treatment in the WiPE are a combination between measurement error, by surrounding algae and because mistaken identification of missing specimens and indicate a growth inhibition.

### 4.3.5. Statistics

Means of photochemistry data ($F_v$:$F_m$) and Y(II) per well were statistically evaluated to avoid pseudoreplication, and consisted of 4-6 individual data points. Photochemistry means were arc sin transformed to achieve normality of the data because it represents proportions. PERMANOVA (Primer v6, Add-on) was performed on transformed data. Temperature has been included as a fixed factor and Aquaria (Temp) were included as a random factor to account for variances between the aquaria. Monte Carlo post hoc tests were performed to test when PERMANOVA was overall significant to establish differences between the individual temperature treatments (Table S2). Growth data (% surface area increase per day) was not statistically evaluated as means per aquaria (n=1-2) did yield a small sample size which did not meet the criteria of normality assumptions.

## 4.4. Results

To investigate the effect of elevated temperatures on *Amphistegina lobifera* and *Pararotalia calcariformata,* both species were initially exposed to a temperature gradient of up to 36°C in an aquarium setup with water movement simulating natural conditions. The experiment was replicated for a population collected in summer (SuPE) and winter (WiPE). This was done to account for the effect of pre-adaptation (hardening) during pre-exposure and is required because of the large seasonal temperature cycle at the studied site (Fig. 1). In addition, a static extreme temperature experiment (ExTE) was performed on *P. calcariformata* in an incubator setup to test its upper thermal limit.

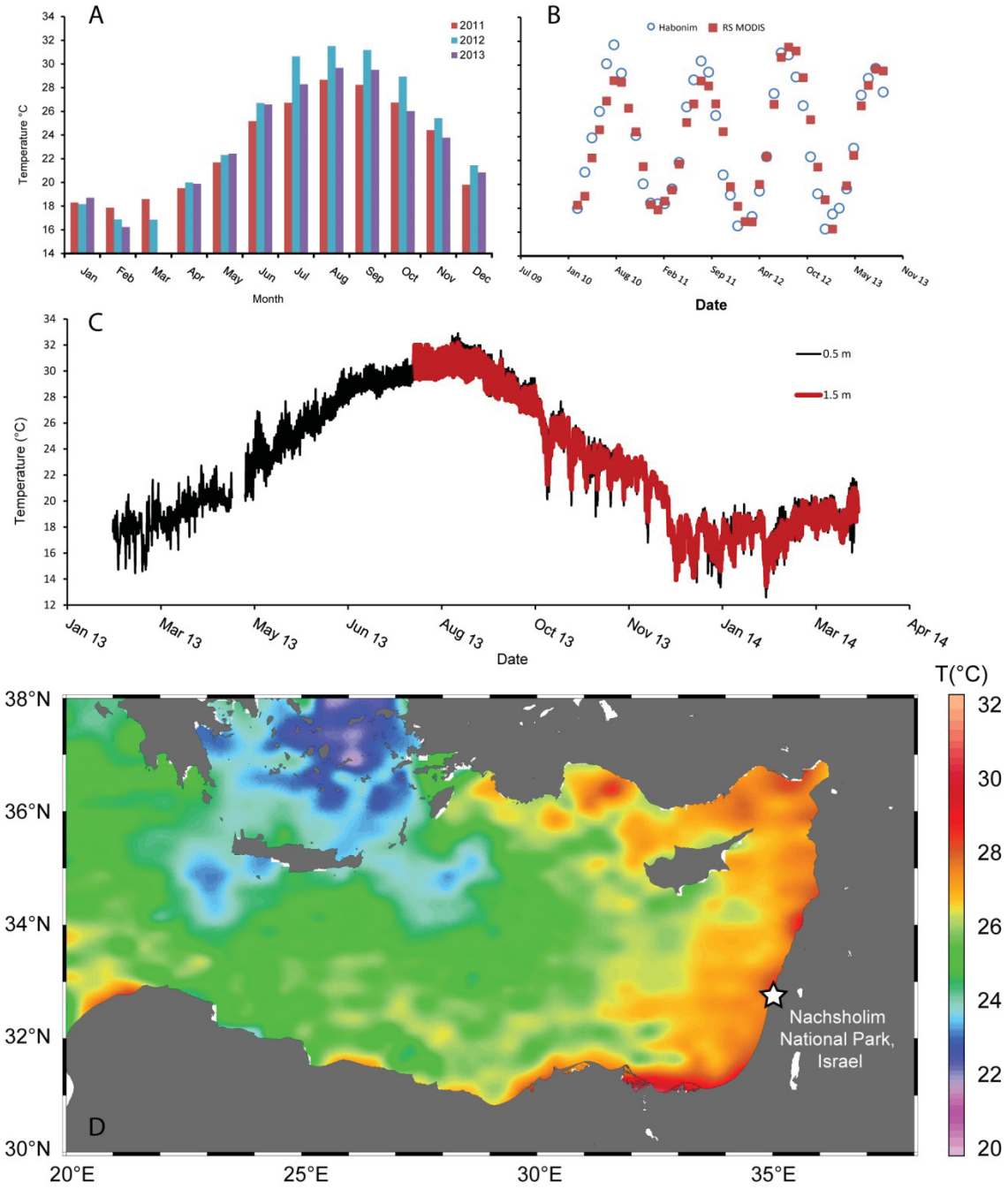


**Figure 1. A) Monthly average SST (2011-2013) Levantine Continental Shelf (up to WD 200m), obtained by Remote Sensing MODIS Data showing an exceptionally warm year in the Levant in 2012. B) Hambonim Station temperature in the Levant (measurements at edge of tidal rocky platform), and Remote Sensing MODIS Temperatures , Source (Herut B. 2014). C) Temperature logger data from the shallow intertidal at Nachsholim National Park (32° 37.386 N, 34°55.169 E, 0.5 and 1.5 m) shows temperatures up to 32°C in August D) Mean summer sea surface temperature in the Levant Basin observed between 1955 and 2012 (July-September) extracted from the World Ocean Atlas 2013 (Locarnini 2013).**

### 4.4.1. Seawater data

The Levant is bathed by the warmest water mass in the Mediterranean (Fig. 1). The region has seen a general warming over the last decades, which is predicted to continue by up to 0.4-2.8°C (Shaltout and Omstedt 2014). Shallow logger data (deployed at 0.5 m and 1.5 m) at the collection site showed that local temperatures in the habitat are higher than predicted from satellite data. Both species are living in the intertidal shallow habitat experiencing 32°C during summer month, so that temperature manipulations up to 35-36°C (SuPE, WiPE) are within global change predictions for the Levant. Control treatments were based on ambient temperatures at the time of collections, 24°C for the summer population experiment (SuPE), and 20°C for the winter population experiment (WiPE) (Fig. 1).

First, in order to investigate the temperature range to which the population from the unpolluted natural site has been exposed to, we analysed climatological data for the Israeli coast and deployed in-situ loggers for two water depth, covering the peak of the warm season (Fig. 1). The coastal waters experience a pronounced seasonal cycle with winter minima around 16°C and peak summer temperatures not exceeding 32°C even during the exceptionally warm year 2012 (Figure 1). The same trend is reflected in the in-situ loggers deployed at the collection site: these indicate short-term exposure at the shallow site during daily fluctuations by no more than 2°C over the long-term monitoring data (Fig. 1). Thus, the studied population is unlikely to have ever been exposed to temperatures above 34°C even during extreme summers. Thus, there appears no reason why the populations should have developed resistance to temperatures of 36°C, as seen in the nearby heat polluted site

### 4.4.2. Symbiont data: Photochemistry

SuPE and WiPE (Summer and Winter Population Experiments): The initial maximum quantum yield ($F_v$:$F_m$) measured of the controls before the start of the SuPE was for *A. lobifera* 0.618, SE ± 0.031) and for *P. calcariformata* 0.555, SD ± 0.029. $F_v$:$F_m$ stayed in the controls (24°C) at the same level from initial to final measurements Fig. 2). PERMANOVA revealed (Table S2) that $F_v$:$F_m$ was not effected by temperature increase during exposure for two weeks up to 35°C. The Y(II) decreased in *A. lobifera* already after one week exposure. A Pair-wise Monte

Carlo test showed that 24-30°C were significantly different from 32 and 35°C. The initial maximum quantum yield ($F_v$:$F_m$) measured of the controls before the start of the WiPE for *A. lobifera* where 0.529, SE ±0.047 and for *P. calcariformata* 0.451, SE ±0.049. PERMANOVA revealed that temperature had a significant effect on the $F_v$:$F_m$ in *A. lobifera* after one week exposure and in *P. calcariformata* after three weeks of exposure (Table S2). The Y(II) was significantly reduced in *A. lobifera* after one week exposure but not in *P. calcariformata*. However, in the Y(II) in *P. calcariformata* a reduced trend from the 34°C to the 36°C treatment is shown (Fig. 2). Photoinhibition occurred ($F_v$:$F_m$ <0.01) under 36°C after one week in the WiPE (60% of *A. lobifera*), and was less drastic than in Y(II) where it was 92% of specimen. After two weeks the $F_v$:$F_m$ increased again in the 36°C treatment to levels of 0.18, SD 0.08, but Y(II) stayed <0.01 in 97% of specimens. A seasonal effect can be seen in the 30°C and 32°C treatments as those were the treatments which where exactly repeated in both experiments. The Y(II) in *A. lobifera*, was higher in the SuPE than in the WiPE. In *P. calcariformata* there is no visible difference in photosynthetic activity to temperatures with season.

ExTE (Extreme Temperature experiment): This experiment was conducted exposing *P. calcariformata* to four different temperatures at 24, 30, 36, and an extreme level of 42°C to determine its upper thermal tolerance level. The $F_v$:$F_m$ and Y(II) of initial symbionts stayed on the same level in the 24°C (control) and in 30°C treatment throughout the experiment (Fig. 3). PERMANOVA revealed that $F_v$:$F_m$ and Y(II) was significantly negatively affected by temperature after one week of exposure (Table S2, S3). Monte Carlo Post hoc tests on $F_v$:$F_m$ and Y(II) indicate that after two weeks there are significant differences between the 24-30°C and the 36°C and 42°C treatments. The reduction in $F_v$:$F_m$ to <0.01 occurred in all specimens of the 42°C treatment and in 14% of the specimen from the 36°C treatment after two weeks.

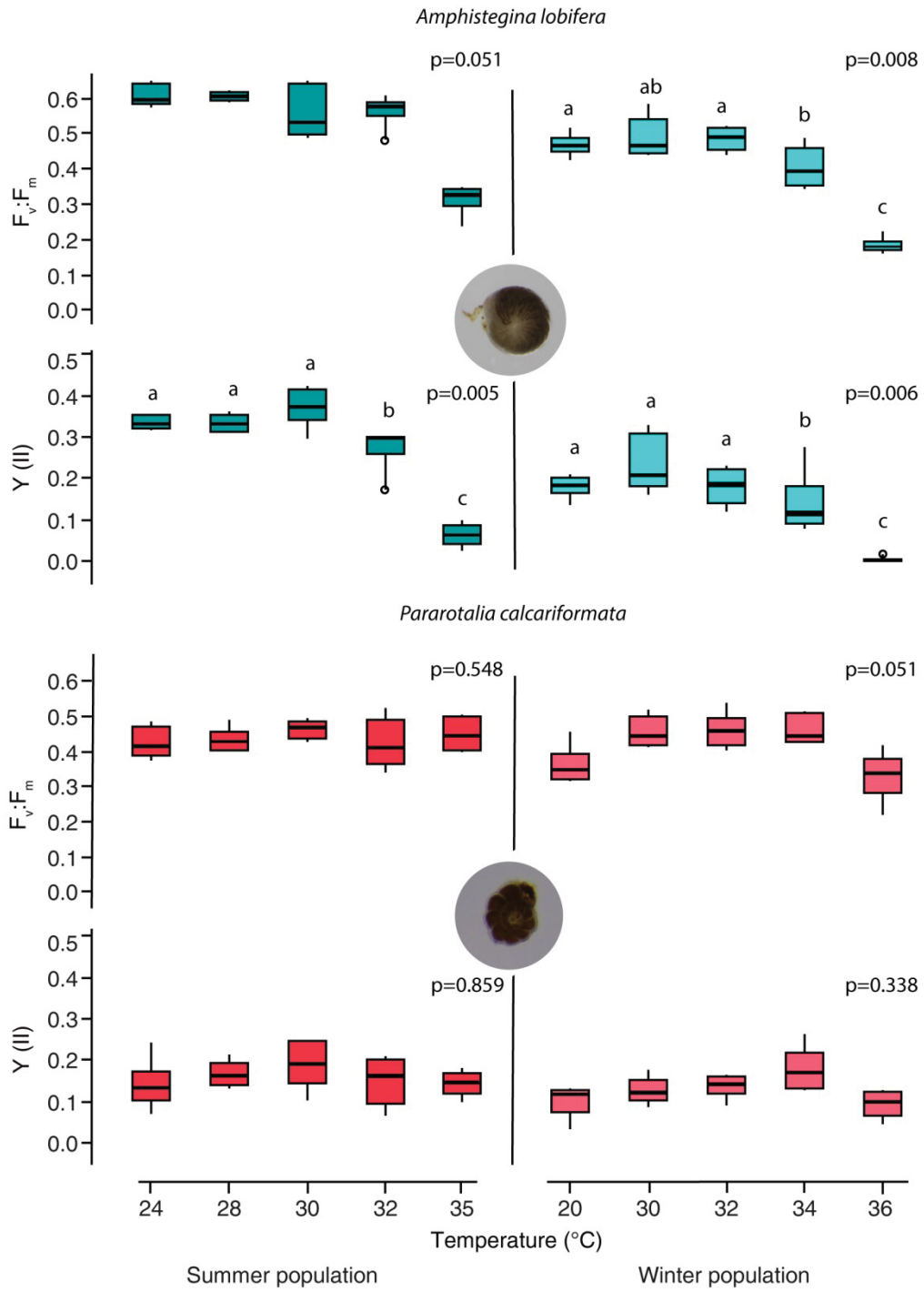


**Figure 2. Pulse Amplitude Fluorometry (PAM) measurements on *Amphistegina lobifera* (blue) and *Pararotalia calcariformata* (red) after two weeks of exposure to experimental temperatures, $F_v:F_m$ represents dark adapted yield, or maximum quantum yield, Y(II) represents light adapted yield or effective quantum yield at 25 µmol photons $m^2$ $s^{-1}$. p values indicate results of PERMANOVA, sig. level α=0.05, different letters indicate sig. differences in the Monte Carlo post-hoc tests between the treatments**

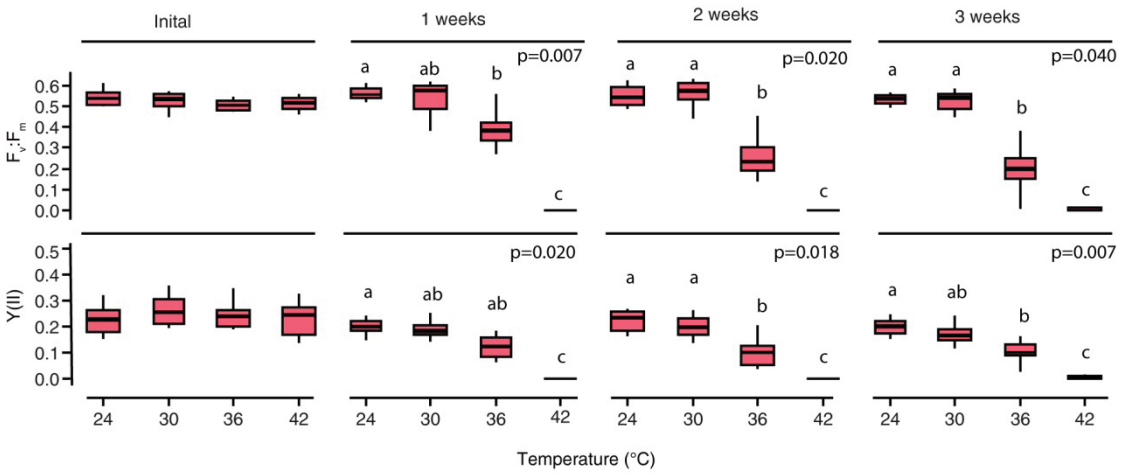


**Figure 3. Pulse Amplitude Fluorometry (PAM) measurements on *Pararotalia calcariformata* initial, after 1, 2 and 3 weeks exposure to elevated temperatures, $F_v:F_m$ represents dark adapted yield, Y(II) represents light adapted yield at 25 µm mol photons $m^2$ $s^{-1}$. p values indicate results of PERMANOVA, sig. level α=0.05, different letters indicate sig. differences in the Monte Carlo post-hoc tests between the treatments**

### 4.4.3. Holobiont data: Survivorship & Growth

After termination of experiments, survival of the holobiont was high, determined by checking of cytoplasmic color. This non-terminal method was chosen, as the species have distinct green-brownish cytoplasm color which can be distinguished from a dead individual, which become translucent upon cell death (Bernhard 2000). Mean survival rates were between 89-100% per treatment (Table S1). Exemplary specimens of *A. lobifera* and *P. calcariformata* in natural habitat and after the laboratory exposure to the respective temperatures for 2-3 weeks (Fig. 4) illustrate that cytoplasmic color was visible in the specimen even after the highest treatments. Paling of shell took place in *A. lobifera* in some specimen in the 34°C, where the expulsion of living or dead symbiotic material is visible (Fig. 4).

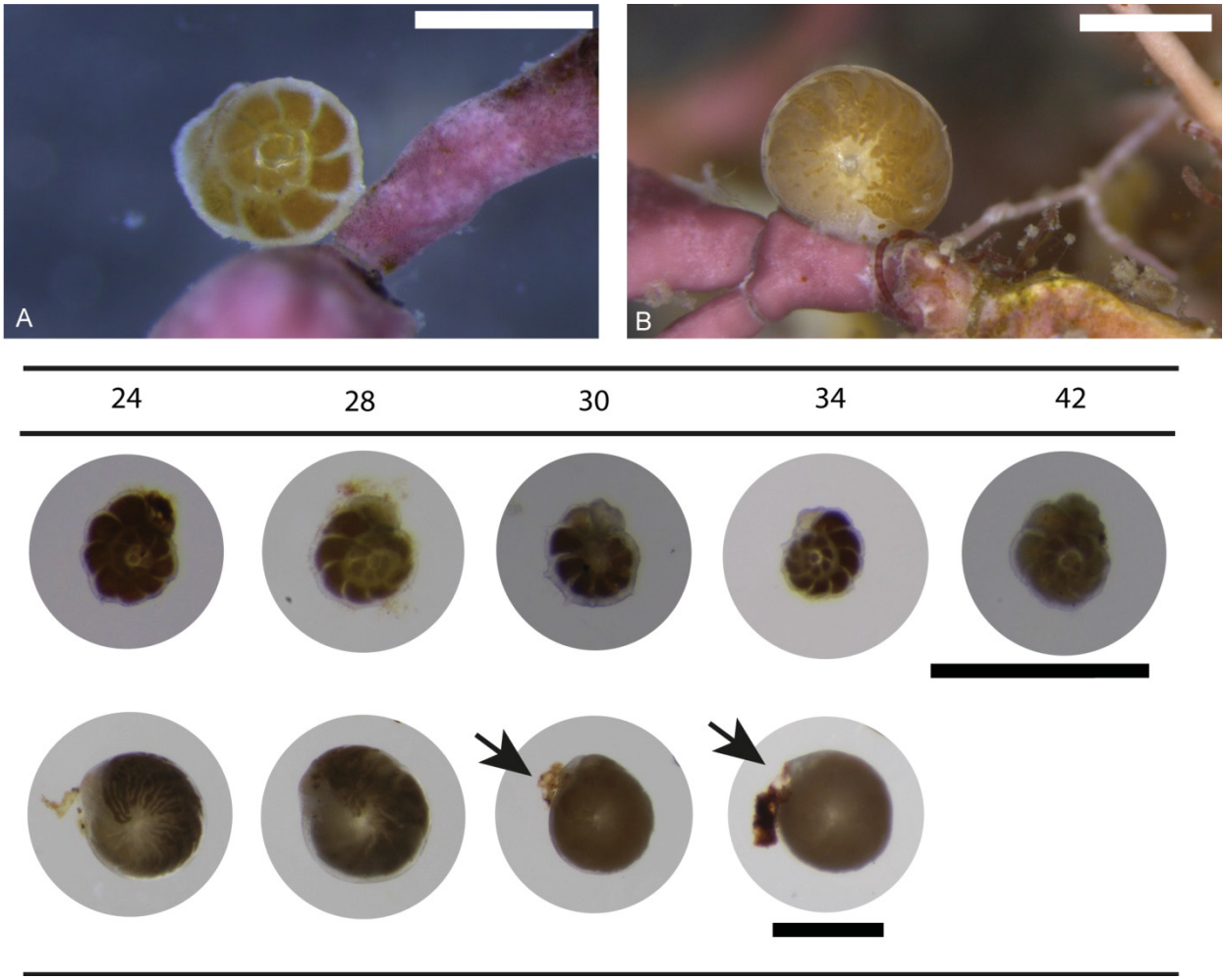


**Figure 4. A, B) Light microscope image of specimens of *Amphistegina lobifera* and *Pararotalia calcariformata* attached to substrate (coralline red algae *Jania sp.*) scale bar: A 0.5 mm, B 1 mm, and C specimens exposed to elevated temperatures in the laboratory for 2-3 weeks, scale bar: 1 mm.**

Positive growth rates (surface area increase per day$^{-1}$ per aquaria) were observed in all the experiments in the control treatments over the experimental period. The summer population (SuPE) of *A. lobifera* showed a near complete growth inhibition at 35°C, whereas *P. calcariformata* still grew under these conditions (Fig. 5). *Amphistegina lobifera* has already reduced growth rates at 30°C compared to the controls, but *P. calcariformata* growth rates under 30-35°C are still in the range of the controls. This indicates a shifted growth optimum between the species. The winter population (WiPE) of *A. lobifera* and *P. calcariformata* grew less than in the experiment using the summer population (Fig. 5). *A. lobifera* did show complete growth inhibition under 32, 34 and 36°C (mean growth rate at ≤0 increase per day).

However, in *P. calcariformata* positive growth rates under 32 and 34°C are still observed. In the extreme temperature experiment (ExTE) the summer population of *P. calcariformata* did generally grew less than in the earlier experiments. *Pararotalia calcariformata* showed very low growth rates in the 24° and 36°C and an inhibition of growth in the 42°C treatment.

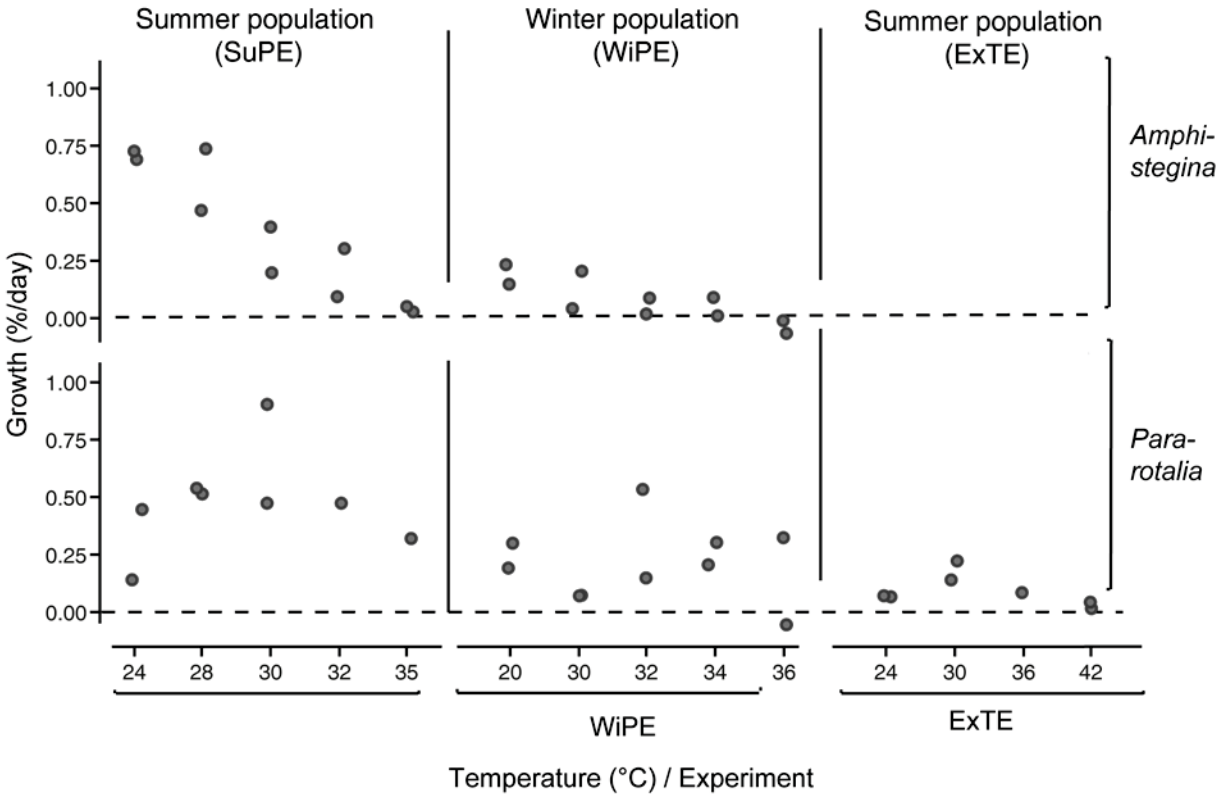


**Figure 5. Growth rates (% surface area increase day$^{-1}$) of *Amphistegina lobifera* and *Pararotalia calcariformata* at the end of the experiment, after two weeks (SuPE) and after three weeks (WiPE, ExTe).**

## 4.5. Discussion

The benthic foraminifera *Pararotalia calcariformata* is able to sustain an active photosymbiosis with diatoms up to 36°C for up to 3 weeks, while *Amphistegina lobifera* collected from the same habitat shows signs of stress under these conditions. Photosynthetic activity was reduced at 34 - 35°C in *A. lobifera*, compared to the controls, but was not in *P. calcariformata*. In *P. calcariformata,* chronic photoinhibition ($F_v$:$F_m$ ≤0.01) occurred at 42°C after one week of exposure in all specimens. Reductions in the $F_v$:$F_m$ represent chronic photoinhibition, defined as the loss of photosynthetic activity due to irreversible photodamage to Photosystem II (Brown 1997). Chronic photoinhibition ($F_v$:$F_m$ ≤0.01) was detected before in LBF in exposed to 33°C for one week (Schmidt et al. 2011). As photoinhibition represents chronic stress in the photosystems, gradual reductions in the light adapted photosynthetic yields Y(II), occurred sooner than reductions in dark adapted photosynthetic yields $F_v$:$F_m$, and are associated with the onset of physiological

consequences of stress inside the organism. In the study by Uthicke et al. (2012), Y(II) was also the more sensitive parameter compared to $F_v$:$F_m$, showing reductions at 34°C after one week and at 31-32°C after one month, as well as reduced oxygen production by the symbionts of the dinoflagellate-bearing *Marginopora vertebralis* (Uthicke et al. 2012). Structural damage and symbiont loss occurred in *Amphistegina gibbosa* under 32°C after one month exposure (Talge and Hallock 2003). In summary, the cellular mechanisms of bleaching are currently not well understood in LBF in comparison to corals (Doo et al. 2014). We can only speculate, that the observations of reduced $F_v$:$F_m$, Y(II) and structural cell damage may result from oxidative stress triggered by reactive oxygen species (ROS) in the cells (Sohal and Weindruch 1996). The activation of the antioxidant systems (measured as ROS concentration) in Amphisteginidae have been observed to counteract oxidative stress but have been demonstrated so far in LBF only in the context of chronic zinc exposure (Prazeres et al. 2011). In the symbionts of shallow-water corals and foraminifera it is widely recognized that the xanthophyll cycle, is another important photo-protective mechanisms, and is able to prevent coral bleaching, (e.g. Warner et al. 1996; Brown et al. 1999; Uthicke et al. 2012). The xanthophyll cycle allows chlorophyll c2 containing algae to deactivate excited chlorophyll by the reversible conversion of diadinoxanthin - diatoxanthin (Demers et al. 1991; Arsalane et al. 1994). The utilization of the xanthophyll cycle in diatoms of *P. calcariformata* could be a possible mechanism to prevent bleaching under temperatures up to 36°C. This remains to be determined as the small size of the specimens and hence little pigment content, prevent direct determination of pigment ratios.

It has been suggested for the coral endosymbiosis (e.g. Bhagooli and Hidaka 2003; Goulet et al. 2005; Visram and Douglas 2007) that the combined physiology of the algae and the host are responsible for the thermal tolerance of the holobiont. Our data show that species can still tolerate extremely high temperatures; 36°C for *A. lobifera* and 42°C for *P. calcariformata* for a short amount of time (<3 weeks) without increased mortality, but are severely negatively impacted under these conditions, as documented by near inhibition of growth rates. Paling of the shell was observed, and expulsion of symbiotic material at the aperture occurred in *A. lobifera* (Fig. 4), similar to earlier studies (Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014). Compared to foraminifera from the Indo-Pacific (Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014), the Mediterranean species seem to be more thermally-

tolerant. This is not surprising, as the seasonal natural temperature exposure the species experience during seasonal cycles is different and likely influences their physiological response in experiments. Background temperatures in the eastern Mediterranean vary from 16-32°C over the yearly cycle (Fig. 1A) and can be elevated by +2°C in the species shallow habitat (Fig. 1C). In the Great Barrier Reef 6 m water depth at Pine Island, where hottest months (January or February 2006-2010) in each year was 28.8°C(1SD = 0.40°C) http://data.aims.gov.au (Uthicke and Fabricius 2012). Specimens of *M. vertebralis* collected close to this station showed that the health of the specimens were negatively impacted at 31°C, and most specimens at 34°C died after one week. *Amphistegina radiata and Heterostegina depressa* from a similar habitat as Uthicke et al. (2012), could survive 33°C for one week but showed disruption of the photochemistry and bleaching (Schmidt et al. 2011).

It has been shown that intertidal invertebrates contain a higher stability form of anti-oxidative enzymes compared to sub-tidal organisms, because they are naturally more adapted to short-term oxidative stress (Regoli et al. 1997). This finding suggests that molecular pre-adaptations also exist between organisms originating from different depth. The organisms from earlier studies originated from habitats in the sub-tidal (Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014) expect *M. vertebralis* in Schmidt et al. (2014) and *Calcarina hispida* in Schmidt et al. (2011) whereas species in our study originate from intertidal environment. Interestingly specimens from the shallow locations in the intertidal 1-2m have also been more resistant towards elevated temperatures. *Calcarina hispida* has had no reduced photosynthetic activity at temperatures of 31°C and *M. vertebralis* showed significant decrease at this temperature but was more resistant than *H. depressa*. Further studies should explore the role of pre-adaptations of depth, and/or symbiont type to in LBF to explore this hypotehesis.

We speculate that the apparently innate resistance to high temperature of *P. calcariformata* originates from the parent populations in the Indopacific. There, in tidal pools of the tropics, it may experience temporary exposure to elevated temperatures and may therefore better pre-adapted. We note that populations of this species exhibiting this tolerance would have had an advantage when passing the shallow coastal areas of the southern Red Sea on their way to invade the Red Sea and later the Mediterranean. Such thermal filtering has been proposed to explain elevated

resistance of corals from the northern Red Sea by Fine et al. (2013). After a dramatic increase in salinity in the Red Sea during the last glacial maximum and local extinction of corals, re-establishment of corals started from the Gulf of Aden, where each larvae had to pass though waters >32°C, at the entrance of the Red Sea (Fine et al. 2013). This could explain the high thermal tolerance of the invaded foraminifera, such as *A. lobifera* and *P. calcariformata*, compared to LBF from the Great Barrier Reef.

We suggest that additional to pre-adaption to higher temperatures of parent populations of the species, special adaptive capacities to the unique thermal tolerance of *P. calcariformata* exist, which explain species-specific differences. The origin of the species cannot be used to prove species-specific differences, as the exact origin of *A. lobifera* population is not clear, until molecular evidence is presented. It has been suggested that it may have recolonized from the Atlantic, is a true “Lessepsian invader”, or has been imported via ballast waters through ships (Langer 2008; Langer et al. 2012). For *P. calcariformata*, we know that a reinvasion through the Atlantic was not an option, as this species has never been described in the Atlantic realm. As *P. calcariformata* has not been identified in the Red Sea, and was not detected in the Mediterranean in earlier assemblage studies (Meric et al. 2008), where new invaders were described and immediately occurred a few years later, we postulate that it is an Indo-pacific invader species (Meric et al. 2008, Chapter 2; Meriç et al. 2013), which could have entered the Mediterranean through ballast waters of container ships or through the Suez Canal after its opening in 1897. Both, hypothesis also explains its greater adaptive capacity to cope with stress. External influences on invasive species such as propagule pressure, new environmental factors, and community interactions could have played a role (Colautti et al. 2006; Occhipinti-Ambrogi 2007).

For the assessment of global change effects on intertidal ecosystems, information on the calcification rates are important because these parameters will determine biodiversity and carbonate production in intertidal environments. Calcification of the species can be easily estimated by growth increases of surface area or diameter (Schmidt et al. 2011). Our study shows a near complete inhibition of growth in *A. lobifera* starting at 35°C in the summer population and at 32°C at the winter population, and in *P. calcariformata* at 36°C. In studies exposing asexual

juveniles of *P. calcariformata* for 48 days to 35°C we found, that juvenile's development is near inhibited under these conditions. This let us conclude that the offspring of *P. calcariformata* are more susceptible to thermal stress than the adult population and require a narrow thermal window for reproduction and ontogenetic development in the range of >20°C and <35°C. The indo-pacific *M. vertebralis* showed also decreased growth at 32°C for up to one month exposure (Doo et al. 2012; Uthicke et al. 2012), indicating that this species has a lower thermal limit than the species in our study. We found slightly different growth optima; for *A. lobifera* between 24-28°C and for *P. calcariformata* between 30-32°C, which are important for judging calcification contribution of the species under global change conditions. This indicates that the environmental envelope of *P. calcariformata* is slightly shifted to the warmer end. Our growth data show that Amphistegenids will be living at the upper limit of their environmental envelope in the Eastern Mediterranean. Their future distribution will therefore be moving towards colder regions in the western Mediterranean as postulated by habitat models (Langer et al. 2012; Weinmann et al. 2013). Amphistegenids, are currently the dominant symbiont-bearing species in the Mediterranean sediments (Meric et al. 2008; Triantaphyllou et al. 2009; Mouanga and Langer 2014), but as their distribution shifts westwards, biodiversity pattern of marine sediments are likely to be altered. Our growth data suggests that *Pararotalia calcariformata*, will be the "survivor species" of climate change in the eastern Mediterranean, as they experience optimal conditions for calcification in waters of 30-32°C and are thus likely to be abundant in marine sediments in the future. Benthic foraminifera have been shown to be of large importance for coral reefs, despite their small size (Scoffin and Tudhope 1985; Langer et al. 1997; Doo et al. 2012; Doo et al. 2014). They can be mass cultured for coastal protection of islands prone to sea-level rise, because of their fast growth and reproduction (Hosono et al. 2014).

Debate has arisen in the coral literature to what extent the genetic variants of *Symbiodinium* symbionts can influence the bleaching response of corals (Iglesias-Prieto et al. 2004; Putnam et al. 2012). For example in the coral *Pocillopora damincornis,* It has been found, that *Symbiodinum* Clade C are less prone to bleaching than *Symbiodinum* Clade D (Glynn et al. 2001; Rowan 2004). Furthermore, bleaching responses are likely shaped by fine-scale differences in symbiont type and go beyond broad cladal designations (Sampayo et al. 2008). In corals the association of symbionts is flexible and characterized by a change of strains over time (Little et

al. 2004) indicating that strain type might have a large influence on the physiology of the holobiont. It has further been shown that photo-symbiont thermal adaptation can change the coral thermal tolerance (Howells et al. 2012). The symbiosis in foraminifera is different from corals, as they have been shown to contain multiple different algae species (Lee and Anderson 1991; Lee 1992). Both species under investigation host a consortium of different diatom endosymbiotic species inside their cytoplasm, which are contained in morphological structures in their calcite shell (Lee et al. 1995; Chai and Lee 1999; Lee and Correia 2005). *Pararotalia calcariformata* host a symbiosis with at least three different diatom symbionts, but also a newly described diatom symbiont, which has not been isolated from a host before, and identified as *Minutocellus polymorphus* (<2-3 µm size) (Chapter 2). *Amphistegina lobifera* has been shown to host up to seven different species of endo-symbiotic diatoms but not *M. polymorphus* (Lee and Correia 2005). We propose that this species could be the clue to its thermal resistance. *Minutocellus polymorphus* grown in batch-culture showed increase anti-oxidant activity (super oxide dismutase activity, SOD) by the factor of 3 at the beginning of its exponential growth phase and had reduced SOD activity at the end of their growth phase, similar to other micro algae (Sigaud-Kutner et al. 2002). This indicates that *M. polymorphus* might be a possible catalyzer, when in its optimal growth phase, for inducing anti-oxidants in the protoplasma *of P. calcariformata* which may reduce shell damage due to thermal-stress. Another theory is that hosting multiple distinct species of symbionts may provide an advantage for the host under rapidly increasing temperatures due to climate change, because it has been shown that not all symbionts provide the same functional benefit to the host (Douglas 1998; Loram et al. 2007). Molecular PCR-based analysis of foraminifera suggests that multiple *Symbiodinium* lineages uniquely associate with foraminifera (e.g., clades F3-F5, G1, H, I) (Garcia-Cuetos et al. 2005; Pochon et al. 2007). Work on the symbiont-bearing *Marginopora vertebralis* showed that dominant types C3 and C15, which are common in corals (e.g. LaJeunesse et al. 2003; Cooper et al. 2011; Putnam et al. 2012) are also found in foraminifera (Momigliano and Uthicke 2013). Almost all hard coral families can host more than one symbiont clade, and molecular work likely underestimated numerical abundances because PCR-based identification techniques still miss cryptic diversity (Baker and Romanski 2007).

Our data highlight the importance of defining thermal tolerance levels on the basis of individual species to make predictions on the basis of species-specific traits to best accommodate for global change. Here, we show that the photosymbiosis of *Pararotalia calcariformata* is more thermally resistant than any other eukaryote-eukaryote symbiosis in invertebrates found to date, as it can tolerate temperatures up to 36°C sustaining a photosynthetically active photosymbiosis over three weeks and can tolerate temperatures of 42°C with damaged photo-symbiosis but not induced host mortality. *Pararotalia calcariformata* hosts at least four different endosymbiotic diatoms, one of which is the newly described *Minutocellus polymorphus* (Chapter 1), which could be a hint towards the higher thermal resistance of the species. As the invasive history of the *P. calcariformata* is not entirely resolved, we can only speculate on possible other mechanisms on its thermal tolerance beside the different endosymbiont. Our growth data shows that *P. calcariformata* currently does not live close to its physiological limits under normal summer temperatures of 32°C in the Levant, and it is likely flourish under 1-3°C warming. Future work needs to test, whether all species of the genus respond similar and determine what role the newly described symbiont plays, in the extreme thermal tolerance of the photosymbiosis in *P. calcariformata*. The need arises for gaining deeper understanding of the functional benefits of endosymbiotic diversity and antioxidant capacity in eukaryotic endosymbionts under thermal stress.

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## 4.7. Supporting Information

**Table S1. Mean Survivorship (%) per treatment during temperature sensitivity experiments exposing *Pararotalia calcariformata* and *Amphistegina lobifera* in for 2-3 weeks to elevated temperatures, n.m.= not measured, as *A. lobifera* was not exposed in this experiment.**

| Experiment | Temperature (°C) | Species | |
|---|---|---|---|
| | | *Pararotalia calcariformata* | *Amphistegina lobifera* |
| 1. Summer Population (SuPE) | 24 | 100 | 100 |
| | 28 | 100 | 100 |
| | 30 | 100 | 100 |
| | 32 | 95 | 93 |
| | 35 | 93 | 90 |
| 2.Winter Population (WiPE) | 20 | 100 | 100 |
| | 30 | 96 | 90 |
| | 32 | 90 | 96 |
| | 34 | 89 | 98 |
| | 36 | 100 | 98 |
| 3. Extreme Temperature (ExTE) | 24 | 100 | n.m. |
| | 30 | 99 | n.m. |
| | 36 | 99 | n.m. |
| | 42 | 99 | n.m. |

**Table S2. Statistical results of PERMANOVA on photochemistry variable Maximum Quantum Yield $F_v$:$F_m$, after dark adaptation, when p was significant at 0.05, pair-wise Monte Carlo test was performed, species names abbreviated, P: *Pararotalia calcariformata*, A: *Amphistegina lobifera*.**

| Experiment | Week | Species | Factor | Maximum Quantum Yield ($F_v$:$F_m$) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Source | df | MS | F-ratio | p | pair-wise Monte Carlo test |
| 1. Summer Population 24, 28, 30, 32, 35°C (SuPE) | 1 | *A* | Temp | 4 | 160.23 | 49.726 | 0.053 | |
| | | | Aq (Temp) | 5 | 3.2222 | 0.4348 | 0.828 | |
| | | | Error | 20 | 7.4108 | | | |
| | | *P* | Temp | 4 | 13.034 | 0.57694 | 0.68 | |
| | | | Aq (Temp) | 5 | 22.591 | 2.3255 | 0.08 | |
| | | | Error | 20 | 9.7147 | | | |
| | 2 | *A* | Temp | 4 | 1220 | 26.46 | 0.051 | |
| | | | Aq (Temp) | 5 | 46.146 | 1.9314 | 0.102 | |
| | | | Error | 19 | 23.892 | | | |
| | | *P* | Temp | 4 | 31.269 | 0.89352 | 0.548 | |
| | | | Aq (Temp) | 5 | 34.995 | 1.0907 | 0.378 | |
| | | | Error | 20 | 32.085 | | | |
| 2. Winter population 20, 30, 32, 34, 36°C (WiPE) | 1 | *A* | Temp | 4 | 5730.2 | 14.984 | 0.004 | A, AB, B, AC, D |
| | | | Aq (Temp) | 6 | 382.42 | 0.74154 | 0.721 | |
| | | | Error | 22 | 515.71 | | | |
| | | *P* | Temp | 4 | 386.46 | 2.7838 | 0.076 | |
| | | | Aq (Temp) | 6 | 138.82 | 6.9128 | 0.003 | |
| | | | Error | 22 | 20.082 | | | |
| | 2 | *A* | Temp | 4 | 2474.9 | 97.944 | 0.008 | A, AB, A, B, C, |
| | | | Aq (Temp) | 6 | 25.268 | 0.87255 | 0.512 | |
| | | | Error | 22 | 28.959 | | | |
| | | *P* | Temp | 4 | 451.63 | 4.595 | 0.051 | |
| | | | Aq (Temp) | 6 | 98.287 | 2.8421 | 0.027 | |
| | | | Error | 22 | 34.583 | | | |
| | 3 | *A* | Temp | 4 | 3017.2 | 67.932 | 0.004 | A, A, AB, B, C |
| | | | Aq (Temp) | 6 | 44.416 | 1.3024 | 0.266 | |
| | | | Error | 22 | 34.102 | | | |
| | | *P* | Temp | 4 | 226.77 | 6.9538 | 0.032 | AB, AB, A, AB, B |
| | | | Aq (Temp) | 6 | 32.856 | 1.6515 | 0.197 | |
| | | | Error | 20 | 19.895 | | | |
| 3. Extreme Temperature 24, 30, 36, 42°C (ExTE) | 1 | *P* | Temp | 3 | 15812.00 | 11.83 | 0.007 | A, AB, B, C |
| | | | Aq (Temp) | 4 | 1336.90 | 1.12 | 0.321 | |
| | | | Error | 40 | 1196.60 | | | |
| | 2 | *P* | Temp | 3 | 18804.00 | 13.98 | 0.020 | A, A, B, C |
| | | | Aq(Temp) | 4 | 1345.40 | 1.01 | 0.430 | |
| | | | Error | 40 | 1332.40 | | | |
| | 3 | *P* | Temp | 3 | 18362.00 | 14.22 | 0.040 | A, A, B, C |
| | | | Aq (Temp) | 4 | 1291.20 | 0.88 | 0.592 | |
| | | | Error | 40 | 1459.20 | | | |

**Table S3. Statistical results of PERMANOVA on photochemistry variable Effective Quantum Yield Y (II), after light adaptation, under experimental conditions, when p was significant at 0.05, pair-wise Monte Carlo test was performed, species names abbreviated, P= *Pararotalia calcariformata*, A= *Amphistegina lobifera*.**

| Experiment | Week | Species | Factor | Effective Quantum Yield (Y(II)) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Source | df | MS | F-ratio | p | pair-wise Monte Carlo test |
| 1. Summer Population 24, 28, 30, 32, 35°C (SuPE) | 1 | *A* | Temp | 4 | 452.1 | 44.401 | 0.024 | A, B, B, AB, C |
| | | | Aq (Temp) | 5 | 10.182 | 1.1013 | 0.413 | |
| | | | Error | 20 | 9.2452 | | | |
| | | *P* | Temp | 4 | 316.92 | 1.7185 | 0.31 | |
| | | | Aq (Temp) | 5 | 184.42 | 1.4121 | 0.291 | |
| | | | Error | 20 | 130.6 | | | |
| | 2 | *A* | Temp | 4 | 5303.8 | 109.39 | 0.005 | A, A, A, B, C |
| | | | Aq (Temp) | 5 | 48.304 | 0.31356 | 0.987 | |
| | | | Error | 19 | 154.05 | | | |
| | | *P* | Temp | 4 | 241.72 | 0.35266 | 0.859 | |
| | | | Aq (Temp) | 5 | 685.41 | 4.9975 | 0.004 | |
| | | | Error | 20 | 137.15 | | | |
| 2. Winter population 20, 30, 32, 34, 36°C (WiPE) | 1 | *A* | Temp | 4 | 6820.2 | 6.4499 | 0.006 | AB, A, A, B, C |
| | | | Aq (Temp) | 6 | 1057.4 | 1.1544 | 0.261 | |
| | | | Error | 22 | 915.95 | | | |
| | | *P* | Temp | 4 | 1186.7 | 3.6021 | 0.065 | |
| | | | Aq (Temp) | 6 | 329.46 | 1.5806 | 0.168 | |
| | | | Error | 22 | 208.43 | | | |
| | 2 | *A* | Temp | 4 | 6745.6 | 6.4406 | 0.006 | A, A, A, B, C |
| | | | Aq (Temp) | 6 | 1047.4 | 1 | 0.453 | |
| | | | Error | 22 | 1047.3 | | | |
| | | *P* | Temp | 4 | 918.26 | 1.2908 | 0.338 | |
| | | | Aq (Temp) | 6 | 711.37 | 3.4386 | 0.007 | |
| | | | Error | 22 | 206.88 | | | |
| | 3 | *A* | Temp | 4 | 7033.6 | 7.986 | 0.018 | A, A, AB, B, C |
| | | | Aq (Temp) | 6 | 880.74 | 1.0274 | 0.426 | |
| | | | Error | 22 | 857.25 | | | |
| | | *P* | Temp | 4 | 967.49 | 3.6015 | 0.056 | |
| | | | Aq (Temp) | 6 | 271.44 | 2.2034 | 0.067 | |
| | | | Error | 20 | 123.19 | | | |
| 3. Extreme Temperature 24, 30, 36, 42°C (ExTE) | 1 | *P* | Temp | 3 | 15901.00 | 9.57 | 0.020 | A, AB, AB, C |
| | | | Aq (Temp) | 4 | 1662.20 | 1.35 | 0.130 | |
| | | | Error | 40 | 1227.90 | | | |
| | 2 | *P.* | Temp | 3 | 18197.00 | 13.23 | 0.018 | A, A, B, C |
| | | | Aq(Temp) | 4 | 1375.20 | 0.96 | 0.499 | |
| | | | Error | 40 | 1433.70 | | | |
| | 3 | *P* | Temp | 3 | 15674.00 | 15.52 | 0.007 | A, AB, B, C |
| | | | Aq (Temp) | 4 | 1009.90 | 0.88 | 0.547 | |
| | | | Error | 40 | 1150.90 | | | |

# 5. Concluding remarks and perspectives

## 5.1. General discussion and conclusions

**Working hypothesis Chapter 2: Combined effects of warming and ocean acidification on coral reef Foraminifera *Marginopora vertebralis* and *Heterostegina depressa.***

**A.) There is a difference between the individual and the combined effects of OA and warming on the physiology of *Heterostegina depressa* and *Marginopora vertebralis***

**B.) The combined effects are additive, synergistic or antagonistic (cancel each other)**

**C.) The response to the combined effects is species-specific**

Results show that the combined effects of ocean acidification and warming will impact the physiology of benthic-symbiont bearing foraminifera *Heterostegina depressa* and *Marginopora vertebralis* stronger than if the stressors would act individually. Overall temperature had been the major stress parameter on foraminiferal physiology, as shown in previous studies (Schmidt et al. 2011; Uthicke et al. 2012; Sinutok et al. 2014), indicating that there was a significant effect of temperature in all parameters measured. However, in many parameters combined effects had a stronger negative effect.

In half of the investigated parameters, which were survivorship, growth, respiration, chlorophyll a content and three parameters describing photosynthesis, significant interactions of OA and warming were found, of which 75% were synergistic (Chapter 1, Table 3). In these cases warming and elevated $pCO_2$ led to a stronger physiological response than the sum of the effects of the individual parameters. We suggest that the energetic costs of counteracting the effect of one stressor might reduce the ability of the holobiont to counteract the effects of an additional stressor. For example the energetic cost for the holobiont to control its symbiotic population under abnormal environmental scenarios such as elevated $pCO_2$ or elevated nutrients has been proposed to lower bleaching thresholds in corals and larger benthic foraminifera (LBF) (Wooldridge 2009; Uthicke et al. 2012).

Few studies have been conducted on the interactive effects of environmental stressors on LBF. They suggest that temperature and nutrient exposure (Uthicke et al. 2012), temperature and OA (Reymond et al. 2013) and temperature and pesticide exposure (van Dam et al. 2012), have interactive or additive negative effects on the LBF. One study presented by Sinutok et al. (2011) on the combined effects of acidification and temperature showed reduced calcification (growth, magnesium and aragonite cystal size) in *M. vertebralis* in the combined treatment. However, in this study the photosynthetic activity and chlorophyll a contents were reduced even in the controls, indicating that LBF might have been severely stressed in this study under culturing conditions, because of extremely high light levels. A second study on the interactive effects of OA and temperatures showed in comparison to healthy controls that the combined effects reduce calcification and photosynthesis in *M. vertebralis* (Sinutok et al. 2014).

The results presented here, indicate that the effects of ocean acidification and warming are likely to be underestimated when stressors are tested isolation. The results prove that responses are species-specific, as the most important synergistic effects were found on different parameters in *H. depressa* and *M. vertebralis*. The latter species showed not effect of the stressor warming or OA on growth alone but was significantly reduced in the combined treatment, where *H. depressa* did not show negative synergistic effects.

**Working hypotheses Chapter 3: Recent invasion of the symbiont-bearing foraminifera *Pararotalia* into the Eastern Mediterranean facilitated by the ongoing warming trend**

A) **Based on its current distribution, the recently discovered foraminifer *P. calcariformata* in the eastern Mediterranean Sea is an invader species**
B) **The foraminifer *P. calcariformata* contains permanent diatom endosymbionts**
C) **The symbionts in *P. calcariformata* are photosynthetically active over several month in culture**
D) **The foraminifer *P. calcariformata* will spread to currently colder regions in the Mediterranean based on global warming trend**

**E) *Pararotalia calcariformata* has a narrow reproductive window for asexual offspring development and is currently restricted by minimum temperatures to spread westwards**

We conclude, from current and past occurrence data, that *P. calcariformata* McCulloch 1977 is likely to be an invasive species in the Mediterranean. We suggest that the invasive population has likely originated from the Indo-pacific region based on taxonomic and phylogenetic investigations. In summary, all evidence suggests that *P. calcariformata* might be a Lessepsian migrant, whose invasion was facilitated by the recent warming in the Levant (Shaltout and Omstedt 2014).

The identity of symbionts in *P. calcariformata* has never been investigated, and the earliest observations of symbionts were described by Arieli et al. (2011). This species harbors functional endosymbiotic diatoms inside its cytoplasm, which we have identified by standard algae culturing and sequencing. We found three different endosymbiotic species inside the host, which are most likely dominated by the endosymbiont *Minutocellus polymorphus*. This species was not observed in any isolated foraminifer before (Lee and Anderson 1991; Lee 2006; Lee et al. 2010) and has been found as a pelagic free-living diatom in the Mediterranean (Sarno et al. 1993).

We measured the photosynthetic activity of symbionts of *P. calcariformata* and conclude that it involves a permanent photo-symbiosis, using PAM (Pulse Amplitude Modulated) Fluorometry, as observed in other benthic foraminifera (Nobes et al. 2008; Ziegler 2009). Rapid light curves indicated that the species might have a stronger light sensitivity than *A. lobifera*, which are in line with findings of Nobes et al. (2008) on *Calcarina* sp. Next, we confirmed the activity and the persistence of the photo-symbionts after collection in culture for up to 5 month using PAM Fluorometry. The photosynthetic activity measured by obtaining the dark adapted yield ($F_v$:$F_m$) are comparable to other symbiont-bearing LBF (Nobes et al. 2008; Ziegler and Uthicke 2011).

Species distribution modelling (SMD) predicts that under current water temperature conditions the Levant is the optimal habitat for *P. calcariformata*. Moderately suitable habitat, representing “typical” conditions for a species, continues along the coast of Syria to southernmost Turkey, where it was most recently reported

by Meriç et al. (2013). Under global warming, the species will spread from the eastern Mediterranean region to the western and northern Mediterranean region, possibly reaching the Ionian Sea by 2100. To construct the SDM, we used yearly minimum temperature, turbidity and irradiance, but minimum temperature was the single most important variable explaining the distribution.

We report asexual reproduction in *P. calcariformata* and growth in manipulative experiments for the first time. The offspring contained a minimum of three chambers when released from the parent and contained symbionts. Subsequent experiments revealed an initial 30-day period of rapid growth, where most of the new chamber development was occurring, followed by a phase of slower growth, where only one or two more chambers where formed. We could prove the hypothesis that juvenile development is restricted to a narrow thermal tolerance window, as we observed a significant difference between growth rates of asexual juveniles and temperature. New chamber formation and “normal” development was only observed at 24°C and 28°C but not at 20°C or 35°C. Under the lower and upper temperature chosen in the experiment, juveniles also survived the experiments but did not showed strong growth. This indicates that for a successful establishment of a population, this species might be dependent on a narrow reproductive window. We conclude that *P. calcariformata* lives at its lower thermal limit in winter conditions in the Levant and prefers warmer spring temperatures for reproduction to ensure optimal offspring development. The Levantine basin has already experienced significant winter warming in the past decades and will increase its yearly mean SST by 0.5-2.3°C providing optimal conditions for the species to (Shaltout and Omstedt 2014). In the Ionian Sea (e.g. San Stefano, Corfu) the time window of temperatures >20°C per year are shorter by two month (IOLR 2010). This mechanism would provide a possible explanation for the restricted Levantine occurrence of the invasive species, as well as for its apparently recent invasion, and current absence in the Ionian Sea. The absence of the limitation to dispersal, due to the removal of physical barriers through anthropogenic influence and even exaggerated through ship traffic, leads to an almost unlimited ongoing range expansion (Langer et al. 2013; Meriç et al. 2013; Weinmann et al. 2013). These results suggest dramatic changes in the structure of Mediterranean ecosystems as a consequence of global change (e.g. Hiddink et al. 2012; Edelist et al. 2013).

**Working hypotheses Chapter 4: Extreme heat tolerance of a foraminifera–diatom photo-symbiosis**

A) ***Pararotalia calcariformata*** **shows a unique thermal tolerance, with symbionts performing photosynthesis at temperatures up to 36°C, which could be an explanation why it occurs in the heat-plume**
B) **The winter population reacts more sensitive than the summer population to elevated temperatures**
C) **Has the foraminifer *P. calcariformata* the same general thermal limit of bleaching of 1-3°C above observed current summer maxima, compared to corals and other eukaryotic photosymbiosis**

The foraminifer *P. calcariformata* exhibits extreme thermal tolerance even in populations living in natural non-heat-polluted environments. The summer population showed active photosymbiosis under 35°C for the duration of 3 weeks which was not significantly different from the controls and positive growth rates under 35°C. This indicates that *P. calcariformata* is adapted to tolerate sustained exposure to temperatures exceeding summer maxima in their habitat by more than 4°C. *Amphistegina lobifera*, collected in the same habitat, showed already reduced photosynthetic activity at 32°C. Some shells of *A. lobifera* (Fig. 4) paled and the individuals expulsed of symbiotic material at the aperture, similar as shown in other studies on LBF (Schmidt et al. 2011; Uthicke et al. 2012; Schmidt et al. 2014). Thermal stress was shown to cause structural damage and symbiont loss in *Amphistegina gibbosa* under 32°C after one month exposure (Talge and Hallock 2003). We can only speculate that observations of reduced photosynthetic yields and structural cell damage may result from oxidative stress triggered in the cells under stressful external conditions, which may lead to the production of free radicals causing damage to membranes (Sohal and Weindruch 1996).

We repeated the experiments on a summer and winter population to account for the effect of pre-adaptation during pre-exposure, which is required because of the large seasonal temperature cycle at the study site which ranges from 16-32°C. In the winter population we measured reduced dark and light adapted yields under 36°C at exposure for 3 weeks. The results point in the direction that the summer population was less sensitive to elevated temperatures than the winter population, and that

natural pre-adaptation to summer maxima, did reduce the negative effect or postpone it until the third week of exposure.

We conducted an extreme thermal exposure experiment elevating temperature above realistic climate change predictions on a summer population in the following year, to test the effect of up to 42°C. The results showed chronic photo-inhibition (dark adapted yields $F_v$:$F_m$ ≤0.01), a complete disruption of the photochemical energy in the reaction centers of PS (Photosystem) II under these conditions after one week of exposure. Despite the stress of the symbionts, the survivorship was high, indicated by green-brownish cytoplasm color. Chronic photoinhibition ($F_v$:$F_m$ ≤0.01) was detected in *Amphistegina radiata* and *Heterostegina depressa* exposed to 33°C for one week (Schmidt et al. 2011), resulting in bleaching. In contrast, *P. calcariformata* apparently survived temperatures, above + 8°C of its thermal adaption to summer temperatures (30-32°C) in the Levant. This indicates a remarkable thermal tolerance for *P. calcariformata*, which is unique in the eukaryote-eukaryote symbiotic relationships. In comparison corals have been shown to bleach under temperatures of up to 26-36°C (Table 1). Bleaching thresholds in corals are dependent on regional pre-adaption (Fitt et al. 2001; Coles and Riegl 2013; Fine et al. 2013), symbiont type (Glynn et al. 2001; Rowan 2004) and are species-specific (Strychar et al. 2004; Strychar and Sammarco 2009). Corals seem to consistently bleach at temperatures 1-3°C above the experienced summer maxima (Coles and Riegl 2013). Since the same appears to apply to other studied LBF, the adaptation threshold of *P. calcariformata* is unique and it remains to be answered why it acquired this adaptation (Fig. 1). Corals in the Red Sea (Fine et al. 2013) and in the Arabian/Persian Gulf (Coles 1988; Coles and Riegl 2013) have been shown to be the most thermally resistant corals worldwide (Wellington et al. 2001). For the Gulf of Aqaba corals, it has been suggested that this unique temperature resistance is due to thermal filtering of larvae, which had to pass a corridor of >32°C in the southern Red Sea to re-colonize the Red Sea from the Indian Ocean, following deglacial sea level rise (Fine et al. 2013).

Possible mechanisms which might make this species in particular thermally tolerant, should be briefly discussed here:

I.) It may originate from a parent population living outside the Levant where the species was or is exposed to higher temperatures. Such environments could be tidal pools or shallow lagoons in the tropical regions of the Indo-pacific. This hypothesis implies that the species is a (Lessepsian) invader species.

II.) The species could have initially established a population in the Levant around the Hadera plume, and gradually became adapted, due to the operation of the thermal power plant of Hadera elevating temperature of 10°C above current summer maxima since the 80's (Arieli et al. 2011). The elevated temperature conditions could have been an advantage for the species which has shown to have difficulty to cope with the lower temperatures in the Mediterranean (Chapter 2). In this case, the species would also be an invader, but its parent population would not need to have the elevated heat tolerance.

III.) The key to the heat-tolerance is in its association with a new symbiotic diatom, which molecular we identified as *Minutocellus polymorphus*, and which has not been found to be associated with LBF before. In corals it has been shown that the pre-adaptation of *Symbiodinium* types to certain climates shapes the resistance of coral hosts to bleaching (Howells et al. 2012) and that fine scale differences in symbiont type change thermal tolerances (Sampayo et al. 2008).

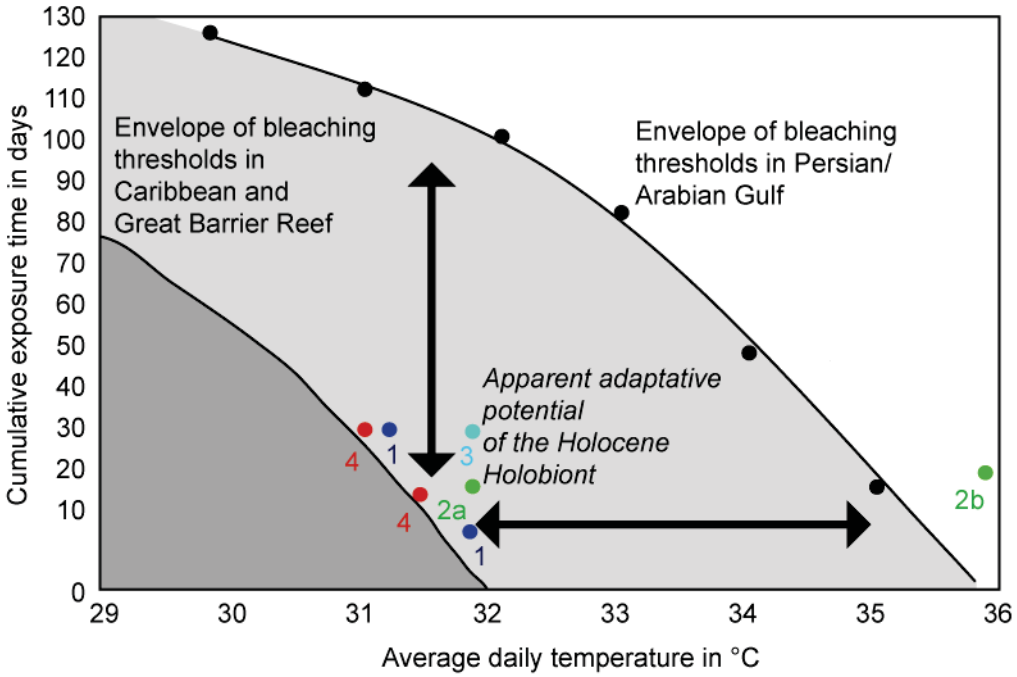
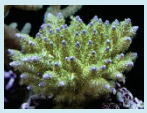
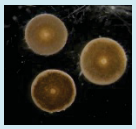
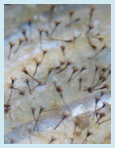



**Figure 1. Bleaching threshold temperature exposure plots for Gulf corals compared with Indo-Pacific and Caribbean corals showing adaptation to the higher water temperatures of the Gulf (redrawn from Coles et al. (2013), to facilitate comparison to thresholds in LBF from these regions and higher adaptation potential of the foraminifera *Pararotalia calcariformata*, coloured dots indicate thresholds from benthic symbiont-bearing foraminifera blue dots: 1 Schmidt et al. (2011, 2014), green dots: 2 a study from Chapter 3 *Amphistegina lobifera*, 2 b study from Chapter 3 *Pararotalia calcariformata*, turquoise dots: 3 Talge & Hallock (2003) *Amphistegina gibbosa*, red dots: 4 Uthicke et al. (2012) *Marginopora vertebralis.***

**Table 1. Marine eukaryote-eukaryote photo-symbioses and thresholds to bleaching of organisms for which symbiosis and/or bleaching was reported.**

| Host | Type | Symbiont Type | Symbiont Reference | Thermal threshold | Bleaching Reference |
|---|---|---|---|---|---|
| **Cnidaria**  | Obligate-obligate | Dinoflagellate *Symbiodinium* Clade C and Clade D in hermatypic corals, soft corals and sea anemones | e.g. Muscatine & Porter (1977), Baker (2003), | 26-36°C +1-3°C above summer maxima, dependent on species and symbiont | e.g. Fitt et al. (2001), Strychar et al. (2004), Fine et al. (2013), Coles et al. (2013), Wellington et al. (2001) |
| **Mollusca**  | Obligate-obligate, kleptoplastidy | Dinoflagellate *Symbiodinium* in marine bivalves and gastropods | e.g. Jones et al. (1986) e.g. Leggat et al. (2003) | >30-32°C | e.g. Addessi (2001), Leggat et al. (2003) e.g. Ziegler et al. (2014) |
| **Rhizaria**  | Obligate-obligate, kleptoplastidy | benthic foraminifera host diatoms, dinoflagellates, red algae and green algae | e.g. Lee et al. (2006) | Benthic Foraminifera >30-36°C, | Hallock et al. (1992), Talge & Hallock (2003), Schmidt et al. (2011) |
| | | benthic Foraminifera *Elphidium sp.* retain diatom plastids (Kleptoplastidy) | Lee et al. (1990), Bernhard & Browser (1999), Pillet et al. (2011) | Not reported | Not reported |
| | | planktonic Foraminifera contain dinoflagellates | Gast et al. (1996) | Not reported | |
| | | Radiolaria contain Prymensiophytes and green algae | e.g. Anderson et al. (1976) | Not repored | Not reported |
| **Ciliates**  | Obligate-obligate, kleptoplastiy | Coral reef ciliate *Maristentor dinoferus* and *Euplotes unicinatus* have dinoflagellates endosymbiont, *Myrionecta rubra* host cryptophyts | e.g. Lobban et al. (2001, 2005), Hansen & Fenchel (2006) | Not reported | Not reported |

## 5.2. Implications of the results: the future of symbiont-bearing foraminifera

The overall results indicate that the temperature tolerance of symbiont bearing foraminifera is species-specific, both for single stressor and combined stressors, confirming observations from earlier studies (Schmidt et al. 2011; van Dam et al. 2012). Our data highlight the importance of defining thermal tolerance levels on the basis of individual species to make predictions including species-specific traits to best accommodate for global change. Similar to corals, determining temperature thermal is difficult as multiple factors such as temperature cycle the population has seen in its lifespan, symbiont factors and also differences between species exist. Furthermore, nutrients, salinity or UV-light (Wooldridge and Done 2009; Reymond et al. 2013) are factors also suggested to influence thermal thresholds and bleaching in LBF (Richardson 2009). Therefore, experiments under future ocean simulations, which can test the influence of more than a single factor at once, provide great potential to evaluate the response to multiple stressors. Further studies should focus on understanding the molecular bleaching response in foraminifera and the role of reactive oxygen species or antioxidant capacities, which could explain species-specific differences (Sohal and Weindruch 1996; Lesser 1997).

The photosymbiont-bearing *Pararotalia calcariformata* has shown to be extremely thermally tolerant, with photosynthetically active symbiosis under 36°C (Chapter 3), showing significantly reduced photosynthetic yields only after three weeks exposure. This threshold together with the apparent survivorship at 42°C exceeds that of the most thermally tolerant corals in the (Persian/Arabian) Gulf (Coles and Riegl 2013). Apparently, the Levantine *P. calcariformata* can survive at temperatures 8°C above the ambient summer maxima (34°C measured in the Levant in 2012 in shallow environment), which make it the most thermally tolerant eukaryote-eukaryote symbiosis found to date. Additional studies on this species should focus on identifying molecular mechanisms of its innate heat resistance, as well as explaining the origin of its adaptation to temperatures exceeding by so much the ecological reality of their habitat. Experimental manipulations on the heat-resistance of the Turkish population described by Meriç et al. (2013), or the Indo-pacific populations from Australia could hint, if this species developed its thermal adaptation in the Mediterranean or is also more thermally tolerant in other locations in the Indo-pacific.

Concerning changes in the microhabitat of LBF global change will lead to changing biogeographic patterns because of invasive species, such as *Pararotalia* (Chapter 2) and *Amphistegina* (Langer et al. 2012; Langer et al. 2013; Weinmann et al. 2013). This together with changes in the macrohabitat influencing their algae substrate it is likely to impact ecosystem function. Invasive species are going to replace native fauna, because if they come from tropical regions they are likely to be better adapted to elevated temperatures and can cope better with global change in regions like the Mediterranean. One prominent example is the invasive macro algae *Caulerpa taxifolia,* which has replaced large parts of the native sea grass *Possidonia oceanica*, and turned a complex three dimensional algae substrate with a longer life-span in a simpler substrate with a shorter life span and so indirectly influenced relative abundance of principal foraminiferal taxa (Mateu-Vicens et al. 2010). Such habitat changes co-occurring with global change could be potential additional stressor to LBF, limiting the population size of already temperature sensitive species. This is likely causing changes on the ecosystem dynamics of the Mediterranean sea, which are going to are impact biodiversity structure (e.g. Hiddink et al. 2012; Edelist et al. 2013) and disturb Mediterranean ecosystem services (Liquete et al. 2013; Katsanevakis et al. 2014).

With regard to ocean acidification and temperature this thesis showed that interactive effects of warming and pH change are going to impact the physiology of the LBF more than the individual stressors alone. We attribute this effect to the reduced energetic capacity of the host to cope with an additional stressor, if it already has to counteract the negative effects of another stressor. Ocean acidification in combination with temperature also negatively impacted growth and photosynthesis in another study (Sinutok et al. 2014). Ocean acidification alone is predicted to have a strong effect on calcium carbonate production, as test dissolution has been documented in experimental manipulations on LBF (McIntyre-Wressnig et al. 2013). Ocean acidification around volcanic $pCO_2$ seeps also caused massive biodiversity changes in foraminiferal communities, giving agglutinated foraminifera a selective advantage compared to calcified taxa (Uthicke et al. 2013).

The central questions we have to ask is if LBF are likely to persist in the future ocean, how we best can understand their adaptive capacity to global change stress and their evolutionary potential to cope with these expected changes in their

environment (Munday et al. 2013). In species which are cultivable over many generations in the laboratory for example phytoplankton, experimental evolution approaches can test their likely response for adaptation in an altered environment (Lohbeck et al. 2013). For benthic foraminifera, the culturing has so far been limited to a 1-4 consecutive generations (Dettmering et al. 1998) and therefore is not an ideal model for adaptive evolution experiments.

In foraminifera, we can try to learn from the fossil record. It has been observed in the fossil record of planktonic foraminifera that shell morphology might have responded to stress before extinction (Weinkauf et al. 2014). Phenotypic plasticity and the ability of a genotype to produce different phenotypes when exposed to different environmental conditions, could play a large role in helping foraminifera to persist under global change. Phenotypic plasticity may also help species to adapt to rapidly changing environmental conditions because it buys time for the slower process of genetic adaptation (Chevin et al. 2010).

In conclusion, we confirmed species-specific differences in the thermal tolerance of symbiont-bearing foraminifera and we identified a photo-symbiosis in benthic foraminifera active under 36°C or higher which is more resistant than most corals and will likely persist under global climate change.

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So eine Arbeit wird eigentlich nie fertig,

man muß sie für fertig erklären,

wenn man nach Zeit und Umständen das Mögliche getan hat.

Johann Wolfgang von Goethe (1749 - 1832),
deutscher Dichter der Klassik, Naturwissenschaftler und Staatsmann

# 7. Schriftliche Erklärung

Gem. § 6(5) Nr.1-3 PromO

**Ich erkläre, dass ich**

**1. die Arbeit ohne unerlaubte, fremde Hilfe angefertigt habe,**

**2. keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe, und**

**3. die den benutzen Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich**

**gemacht habe.**

Bremen, den 30.01.2015

X

Christiane Schmidt