# LONG EXPOSURE OF THE INDO-PACIFIC CORAL *CAULASTREA FURCATA* TO PRESUMED SUBLETHAL TEMPERATURES

**MSc THESIS FINAL REPORT**

J.R. RAIJMAKERS

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# Abstract

Coral reefs around the world have experienced bleaching events in the past decades. Due to climate change, the frequency and intensity of these bleaching events have increased in the last couple of years. Consequently, coral coverage and diversity have decreased, resulting in hampered, less healthy coral reef ecosystems. Therefore, marine life, livelihoods, and coastal protection declined. Despite the devastating effects of coral bleaching, the exact mechanism by which heat disrupts the coral-algal symbiosis remains unconfirmed. Two types of bleaching are presumed: Type I is lethal for the symbiont, and Type II is sublethal. The objective of this study was to gain knowledge into how resilient the Indo-Pacific coral *Caulastrea furcata* is to long exposure at a presumed sublethal temperature. Lethal is defined as the temperature at which the photosystem of the symbiont crashes. Sublethal is the temperature at which the coral-algal symbiosis becomes unstable, leading to an expelling of healthy symbionts. An experiment was conducted to test the hypothesis that *Caulastrea furcata* exhibits Type II bleaching when exposed to the presumed sublethal temperature of 31°C. The symbiosis between the coral host and the symbionts was expected to get disrupted, and the symbiont photosystem remained in good condition at 31°C. The temperature was slowly and gradually increased by 1°C per four days from 26°C to 31°C to simulate a natural bleaching event. After eight days at 31°C, the corals started to bleach, and within five days, the effective yield of the symbionts crashed to zero. Also, the symbiont density declined by 90% during the bleaching process. In conclusion, no clear evidence was found that *Caulastrea furcata* exhibits Type II bleaching at 31°C. This could be explained by the dominant symbiont type *Cladocopium sp.* inside the corals. *Cladocopium* inhabits corals from temperate Japan to the Great Barrier Reef, so it is adapted to a wide temperature range, including heat and cold tolerance. So, *Cladocopium* could be better adapted to relatively cooler sea surface temperatures compared to the symbiont type *Symbiodinium* present in corals used in past experiments, resulting in bleaching at a slightly lower temperature.



Keywords: Coral bleaching, heat stress, climate change, *Caulastrea furcata*, *Cladocopium*, Indo-Pacific.

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# 1. Introduction

## *1.1. Background*

Coral reefs are important ecosystems for the world's oceans and the people whose livelihoods depend on them (Lough & van Oppen, 2018). Although the total area of coral reefs is less than 0.1% of the ocean surface, they support 25% of all marine life (Takagi et al., 2023) and feed hundreds of millions of people through fisheries (Hoegh-Guldberg et al., 2017). Furthermore, coral reefs protect coastlines from hurricanes and other natural disasters by attenuating waves and acting as a natural physical barrier between land and open ocean (Pascal et al., 2016). Lastly, 30% of the world's coral reefs are valuable to the tourism sector, with an estimated total value of US$36 billion (Spalding et al., 2017).

Unfortunately, coral reefs are in decline worldwide (Eddy et al., 2021), which has consequences for the ecosystem services that coral reefs provide. Without a suitable reef habitat, coral reef fish will disappear, causing a cascading effect on species higher up the trophic levels (Sherman et al., 2023). This causes reduced income from fisheries and tourism. Also, due to declining coral reefs, reduced coastal protection is causing higher costs after storms (e.g. damaged houses, infrastructure, etc.).

One of the main causes of the decline of coral reefs is increasing sea surface temperatures due to climate change. Climate change has resulted in multiple mass coral bleaching events worldwide in the past years due to increased heat stress (Eakin et al., 2019). However, if the Paris Agreement of 1.5°C global warming is achieved, coral reefs might bounce back, preventing the mass extinction of coral species and coral reef-related species (Couce et al., 2023; Lachs et al., 2023).

Research has been conducted to study the possibilities of restoring the world's reefs through asexual (Barton et al., 2017; Bayraktarov et al., 2020) and sexual reproduction (Baria-Rodriguez et al., 2019; Flint & Than, 2016; Harrison et al., 2021). Also, NGOs worldwide are actively restoring the world's reefs by fragmenting corals, growing them in nurseries and planting them out on the reefs. However, this restoration work will fail if climate change is not addressed. Also, research is conducted to study the process and mechanisms of coral bleaching. This research can help advocate policymakers to implement climate change mitigation measures to prevent or at least reduce coral bleaching events.

Corals live in symbiosis with unicellular dinoflagellate algae belonging to the family *Symbiodiniaceae*. These symbionts live in the cells of the coral and provide the coral's colour and up to 90% of the coral's energy (Gierz et al., 2017). The process of coral bleaching occurs when this symbiosis becomes unbalanced due to heat stress, resulting in the loss of these symbionts (Weis, 2008). As a result, the coral loses its colour and primary energy supply. Most corals will die of starvation after bleaching (Maire et al., 2022). Two types of bleaching are presumed: Type I is lethal for the symbiont, and Type II is sublethal. Lethal is defined as the temperature at which the symbiont photosystem crashes (Type I). Sublethal is the temperature at which the coral-algal symbiosis becomes unstable, leading to the expelling of healthy symbionts (Type II). So, lethal is not necessarily a temperature that is directly lethal for the coral itself, only for the symbiont.

The exact coral bleaching mechanisms are not yet fully understood. Oakley & Davy (2018) proposed multiple mechanisms of coral bleaching. Firstly, expulsion (exocytosis) of healthy symbionts from the coral host. Heat stress causes more viable symbionts to be expelled from the host cells. Secondly, the degradation or consumption of the symbionts within the host cells (symbiophagy). Heat stress induces disruption or activation of the host's innate immune response, resulting in symbiont consumption by the coral host. Also, it can cause in situ degradation of the symbionts. Thirdly, detachment of healthy host cells containing the symbionts. This detachment might be due to high- and low-temperature stress, but chemically induced stress is also a possible cause. Finally, symbionts can be lost due to the death of the host cell. This can be initiated by severe heat stress in both host and symbiont cells and by reactive oxygen species (ROS) stress.

The family *Symbiodiniaceae* has multiple genera (e.g. *Symbiodinium*, *Breviolum*, *Cladocopium*, and *Durusdinium*), resulting in a large variety of symbiont species (Liu et al., 2018). Each species has slightly different characteristics, meaning they can behave differently when exposed to long-term heat stress. The genus *Durusdinium*, in particular, might act more selfishly and parasitically during heat stress, probably resulting in the host actively kicking out the symbionts. However, this genus is also better capable of withstanding heat, so bleaching occurs later (Stat & Gates, 2011).

## *1.2. Knowledge gap*

Research on which mechanism of coral bleaching is occurring under which conditions is lacking. In short-term heat stress experiments on shallow water specimens of *Cyphastrea serailia* and *Pocillopora damicornis*, Ralph et al. (2001) found that symbionts are expelled by the coral host when the temperatures are too high. Often, this mechanism is suggested to occur in situ during natural bleaching events, although it is not observed regularly. Studying the fate of the symbionts during a real-life scenario (i.e. long-term, gradual heat exposure, simulating a natural summer heatwave) in the lab will aid in providing the necessary understanding of the coral bleaching process.

During previous experiments and studies in Wageningen, it was observed that in multiple long-term (4-6 weeks) bleaching experiments on different coral species, the effective yield of the photosystem within the symbionts, from now on called effective yield, crashed at a temperature of 32-33°C. This indicates that the symbiont's photosystems get damaged at this temperature, leading to their removal from the coral host. Nevertheless, in nature, many corals bleach at temperatures slightly below 32°C (Wijgerde et al., 2020). This discrepancy made us hypothesise that at long exposure to these slightly lower temperatures, coral bleaching is not a result of dysfunctional photosynthesis but rather of a dysfunctional symbiosis, as was also suggested by Rädecker et al. (2021). Rädecker et al. (2021) found that the symbiont density in corals increases at a temperature just below the bleaching threshold, probably due to the more selfish behaviour of the symbionts. Due to the increased temperature, respiration of the symbionts increases at the expense of translocation of photosynthates to the host. This causes the coral host to digest its own protein pool, leading to ammonium production that becomes available to the symbionts. This causes the (normally nitrogen-limited) symbionts to invest in cell division rather than feeding their host. Ammonium production by the coral host is a reaction to the decline of the food supply of the symbionts to their host, resulting in a positive feedback loop where the symbionts feed themselves and can divide their cells (Gardner et al., 2017).

In this experiment, the stony coral *Caulastrea furcata* was exposed to a presumed sublethal temperature. Its bleaching response was monitored through the effective yield, symbiont density, frequency of dividing symbionts and the green fluorescent protein (GFP). Furthermore, the dominant symbiont type was identified to understand the bleaching response better.

## *1.3. Objective & research questions*

The main objective of this experiment is to gain more knowledge on the response of Indo-Pacific corals to long exposure to a presumed sublethal temperature. Another aim is to distinguish what exactly happens with the symbiosis between the coral host and the symbionts under prolonged exposure to heat stress, whether Type I or Type II bleaching occurs. Therefore, the main research question is:

**How does long exposure to a presumed sublethal temperature stress affect the symbiont photosystem and the symbiosis between the coral host and symbionts?**

In addition, two sub-questions are formulated:

- Do the symbionts get killed or expelled due to the heat stress?
- Can eventual differences in bleaching response be related to symbiont identity?

## *1.4. Hypotheses*

To answer this main question, I monitored the effective yield of the symbionts to see whether it would crash during long exposure to a presumed sublethal temperature. Based on previous experiments and studies, it was presumed that 31°C was a sublethal temperature for the symbionts. So, it was expected that *Caulastrea furcata* would exhibit Type II bleaching when exposed to 31°C. As a result, it was expected that the coral-algal symbiosis would have become unstable and the symbiont photosystem would remain in good condition (Type II) rather than that the symbiont photosystem would crash (Type I).

Regarding the first sub-question, Type II bleaching would lead to healthy symbionts being expelled but not killed after the coral-algal symbiosis became unstable due to heat stress. So, the effective yield was expected to remain good and not crash after a prolonged stabilisation. Furthermore, the symbiont density was expected to increase under presumed sublethal conditions due to ammonium production by the coral host (Rädecker et al., 2021), and therefore, also increase the frequency of dividing symbionts, which were not yet expelled. In addition, the GFP was expected to increase and become more visible once the coral bleached and the symbionts were expelled (Roth & Deheyn, 2013).

Regarding the second sub-question, the *Caulastrea furcata* corals used for this experiment originated from Guam in the Indo-Pacific. So, *Cladocopium* was expected to be the dominant symbiont type due to its occurrence from temperate Japan (Lien et al., 2012) to the Great Barrier Reef (Beltrán et al., 2021). Therefore, this genus is adapted to a wide temperature range. *Symbiodinium* was used for most previous bleaching experiments, in which corals always bleached at 32°C or 33°C. So, it was expected that the bleaching response of *Cladocopium* would be slightly lower than *Symbiodinium* due to its origin.

# 2. Materials & Methods

## *2.1. Preparations*

Coral fragments of *Caulastrea furcata* were collected from the University of Oldenburg in Germany. They were placed in the Indo-Pacific coral tank at the Carus research facility at Wageningen University & Research 11 weeks before the start of the experiment. The corals had already been fragmented at the University of Oldenburg into single or double polyps, glued to a concrete foothold, and placed in a plastic spacing grid. The corals were exposed to a natural simulated heat wave in a specifically designed aquarium setup for bleaching studies (Wijgerde et al., 2020; see the following paragraph for details). Corals were acclimated to this setup for two weeks at ambient temperature (26°C) before the heat wave commenced.

## *2.2. Experimental design*

The experimental design (Fig. 1) consists of two basins, a heatwave (basin 1) and a control basin (basin 2). Each basin contained eight independent, replicate 8-litre tanks to prevent contamination. The water inside the tanks did not mix with the water inside the basins. In each basin, a 300 W heater (Schego) was placed and connected to a digital TS125 thermostat (H Tronic, Germany) to set the correct water temperature and a TK150 Aquarium Cooler (TECO) or TK500 (TECO) was placed to keep the temperature constant. Also, each basin was equipped with two 8000 L/h Turbelle nano stream 6085 circulation pumps (Tunze Aquarientechnik GmbH, Germany) to create a circular water flow to distribute the heat equally across the basins. Regarding lights, eight 190 W LED lights (Philips CoralCare) were used to keep the light intensity at 300 µmol $m^{-2}$ $s^{-1}$, and a 12h:12h light-dark cycle was applied. The heights of the lights differed depending on their position above the tanks to create an evenly distributed light intensity. In addition, to prevent a tank from receiving too much heat by standing next to the heater, the tanks were moved weekly by one position.

Six coral fragments were placed in each tank, so 96 corals were used in total. A plastic spacing grid was cut to create a 7x4 plastic grid for each tank where the six corals are systematically placed with two spaces (2 cm) in between the corals (Fig. 2). Each tank was equipped with an EHEIM compactON 300 pump to create a current within the tanks.

Artificial seawater (ASW) was made 48 hours in advance with Zoo Mix artificial sea salt (Tropic Marin GmbH, Germany), deionised water and added nutrients: 15 ml of 0.5 M $NH_4Cl$ and 3 ml of 0.2 M $Na_2HPO_4$. Continuous water refreshment was ensured with a rate of 0.2 ml per minute by two 8-channel peristaltic pumps (Masterflex™) and flexible plastic tubes, which led into the tanks and refreshed each tank with ASW by 50% daily. Each tank was cleaned weekly, and depending on the turbidity of the water in the tank, 75% of the water was manually refreshed weekly to minimise algae growth. The new water was prepared and brought to the correct temperature before refreshment. Furthermore, the coral fragments were carefully cleaned with a toothbrush each week. The temperature and salinity of each tank were monitored daily with a conductivity meter (WTW, Germany). Water quality, including alkalinity, calcium, phosphate, and nitrate, was measured three times a week using PO4 and NO3 Pro test kits (Red Sea Fish) and alkalinity and calcium test kits (Salifert). After the acclimatisation at 26°C, the temperature was gradually increased by 1°C every four days until the temperature was at the targeted, presumed sublethal temperature of 31°C. The sublethal temperature phase of the experiment was planned to last a maximum of six weeks. The corals were fed daily with 1 ml of brine shrimp (*Artemia salina*) per tank at a concentration of 250 individuals $L^{-1}$.

After the corals bleached, the temperature was decreased back to 26°C. Half of the remaining heatwave and control corals were moved to the main holding tank in the lab, where all the other corals are stored to monitor eventual differences in the recovery between the heatwave corals in the original experiment tanks and inside the main tank.

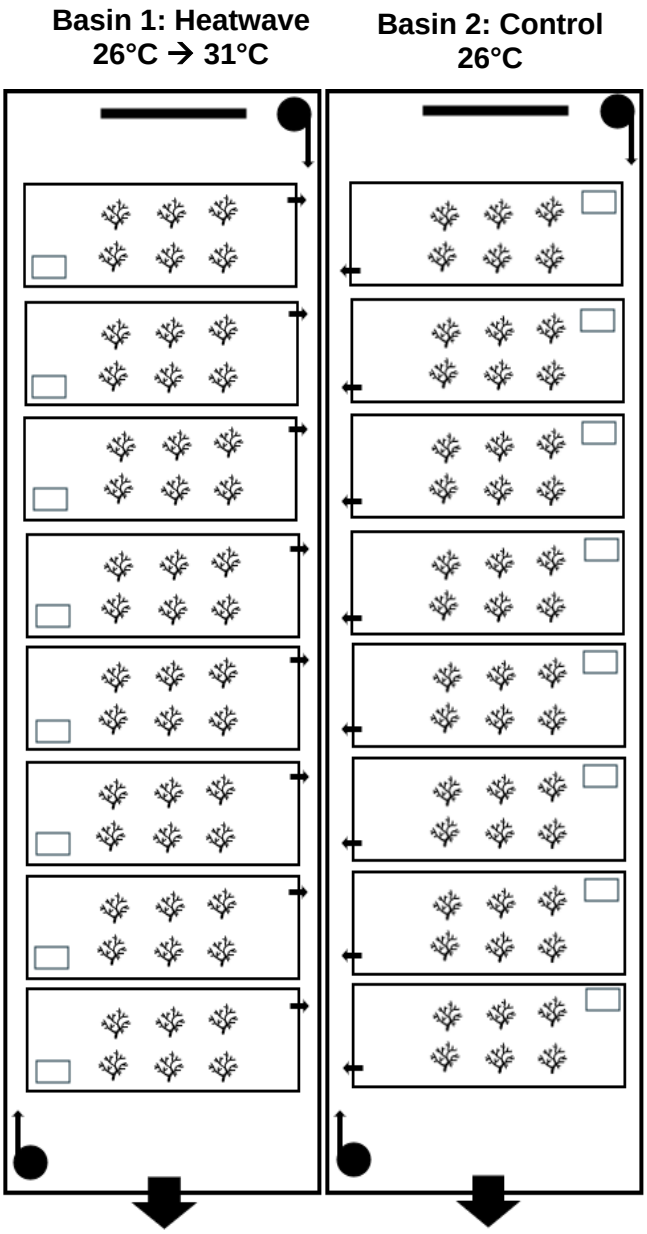


Fig. 1. Overview of the experimental setup. The two large rectangles represent basin 1 and 2. The black bars on top represent the heaters. Black circles indicate circulation pumps with flow direction and the thick filled black arrows below indicate the outflow. In the tanks, the small rectangles indicate the pumps and the small black arrow the outflow. The inflow in the tanks is via a flexible plastic tube which enters the tank through the outflow tube.

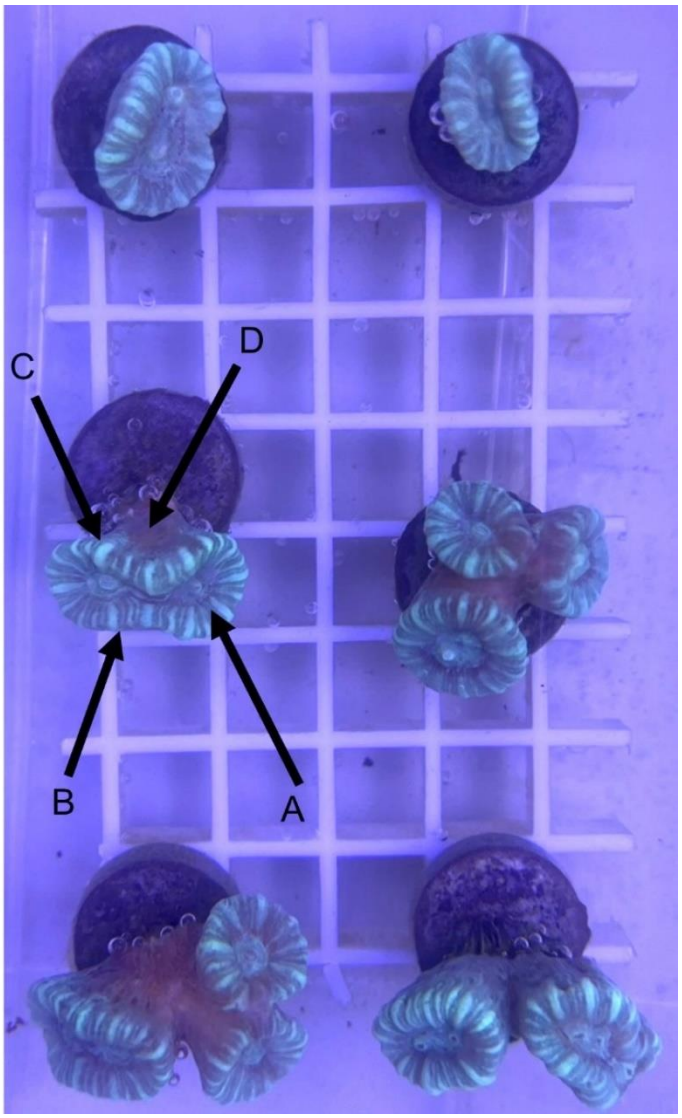


Fig. 2. Six corals per tank in the plastic grid. Arrows indicate the four effective yield measurements. A: on top of the rim, B & C: sides of the rim, D: against the stem of the coral.

## *2.3. Effective yield, symbiont density & Symbiodiniaceae sequencing*

### *2.3.1. Effective yield*

The effective yield of the symbionts is a qualitative measurement. It was measured with Pulse Amplitude Modulation (PAM) fluorometry (Wijgerde et al., 2020). A MINI-PAM-II (Heinz Walz GmbH, Germany) was used to measure the effective yield daily one hour after the lights turned on (between 10:00 and 12:00h). One random coral of each tank was measured at four sites at 2 mm from the surface of the coral.

One measurement was on top of the rim of the coral, two measurements at the side of the rim, and one at the bottom against the stem (Fig. 2). After the bleaching, the effective yield was measured weekly for four weeks to monitor recovery.

### *2.3.2. Symbiont density & frequency of dividing symbionts*

The symbiont density and frequency of dividing symbionts were monitored. This was done following the Protocol Symbiont Density Measurement (Appendix 1). This protocol includes removing the coral tissue with the Waterpik method, centrifuging the tissue, and taking pictures of subsamples of the resuspended pellets with the EVOS Cell Imaging System. Afterwards, these photos were analysed using ImageJ. This entailed counting symbionts and calculating the percentages of dividing symbionts. During the experiment, the symbiont density in the corals was measured three times. The first measurement was during the temperature ramping period on day 30, and the other two measurements were taken during bleaching (days 42 and 44). Four random corals of the heatwave and four random control corals were sacrificed and analysed for each symbiont count. In addition to the three counts during the experiment, one symbiont count was conducted four weeks after the corals were bleached to monitor recovery. However, only two heatwave corals and two control corals were used for this symbiont count.

### *2.3.3. Green fluorescent protein (GFP)*

The relative amount of green fluorescent protein (GFP) in the coral tissue was measured during the bleaching event (days 42 and 44). Coral tissue was removed following the same protocol as mentioned above, and in total, 400 µL (2 x 200) of the supernatant of each coral was pipetted on a 96-well plate. This was then put into the CLARIOstar Plus Microplate Reader to analyse the supernatant for GFP. Lastly, the values were divided by the coral surface area.

### *2.3.4. Symbiodiniaceae sequencing*

The Protocol Symbiodiniaceae Sequencing (Appendix 2) was used to sequence and identify the symbionts in the coral (Hume et al., 2015). Steps included DNA isolation with DNA Power Soil Pro Kit, the amount of DNA checked with Nanodrop, and the PCR product put on gel. The three best samples were sent to Eurofins Agro Testing Wageningen for Sanger sequencing. These samples include one control coral and one heatwave coral, both sacrificed three days after bleaching and one heatwave coral sacrificed during the recovery phase, almost a month after the bleaching started. The first two samples were stored in a fridge for a few weeks, whereas the last was the freshest. The results of the Sanger sequencing were then entered into the BLAST website of the National Center for Biotechnology Information to compare and identify the symbiont species from the sequences.

## *2.4. Statistical analyses*

RStudio version 4.2.1 was used to analyse the data. Each tank consists of six corals (pseudo-replicates), so each tank was considered an independent experimental unit. Therefore, the measured effective PSII yields were averaged per tank. All the data were first tested for normality and homogeneity of variance. This was done using the Shapiro-Wilk test and Levene's test, respectively. Regarding the effective yield, due to the clear results of when the bleaching began, individual two-sample t-tests were conducted to compare the heatwave and control groups on six specific days during the bleaching (days 40, 41, 42, 43, 44, and 45). In addition, a Bonferroni correction was applied to account for the use of multiple t-tests. The data for symbiont density was analysed with a two-way mixed ANOVA, taking time and treatment into account as factors. Furthermore, multiple comparison tests were conducted to determine which groups were significantly different from each other. Also, a two-way mixed ANOVA and multiple comparison tests were performed for the GFP data.

# 3. Results

## *3.1. Effective yield*

### *3.1.1. Bleaching*

The effective yield of the corals in the heatwave treatment started to decline on day 40, whereas the control corals averaged around 0.6 (Fig. 3). On day 40, the heatwave treatment had been exposed to 31°C for eight days. Multiple t-tests were conducted to test if the effective yield of the control and heatwave treatment were significantly different (Appendix 3.1). From day 40 onwards, the effective yield of the control and heatwave treatment differed significantly on all days. On day 41, the effective yield declined by 20% compared to the control group. On day 42, it decreased by 30%, on day 43 by 36%, on day 44 by 78%, and finally, on day 45, the effective yield had declined by 89%.

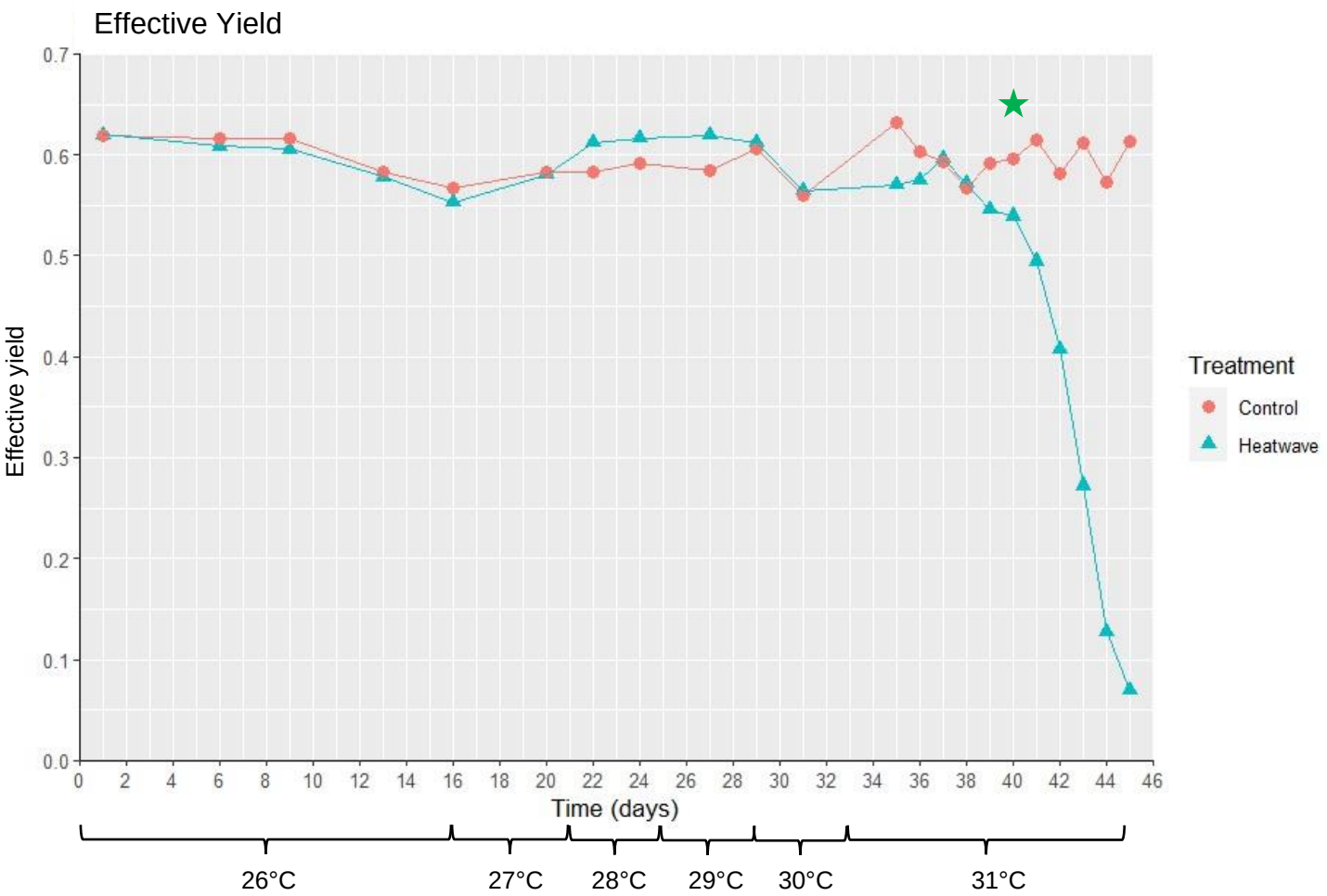


Fig. 3. The effective yield of the control (red circles) and heatwave treatment (blue triangles). The green star indicates when the corals started to bleach (day 40).

### *3.1.2. Recovery of effective yield*

The effective yield did not recover for four weeks after the corals were bleached. The first recovery measurement was one week after the bleaching, the second two weeks, the third three weeks, and the last effective yield recovery measurement was done four weeks after the bleaching started.

During the first three weeks, the effective yield value remained zero. At the last measurement, the effective yield was 0.239 in the heatwave tanks and 0.146 in the main holding tank. So, the bleached corals that stayed in the heatwave tanks and those placed in the main holding tank did not show recovery during this period.

## *3.2. Symbiont density*

### *3.2.1. Bleaching*

The symbiont density was measured at three moments during the experiment (Fig. 4). The first measurement was on day 30 during the temperature ramping to create a baseline. The other two moments were during the bleaching on days 42 and 44.

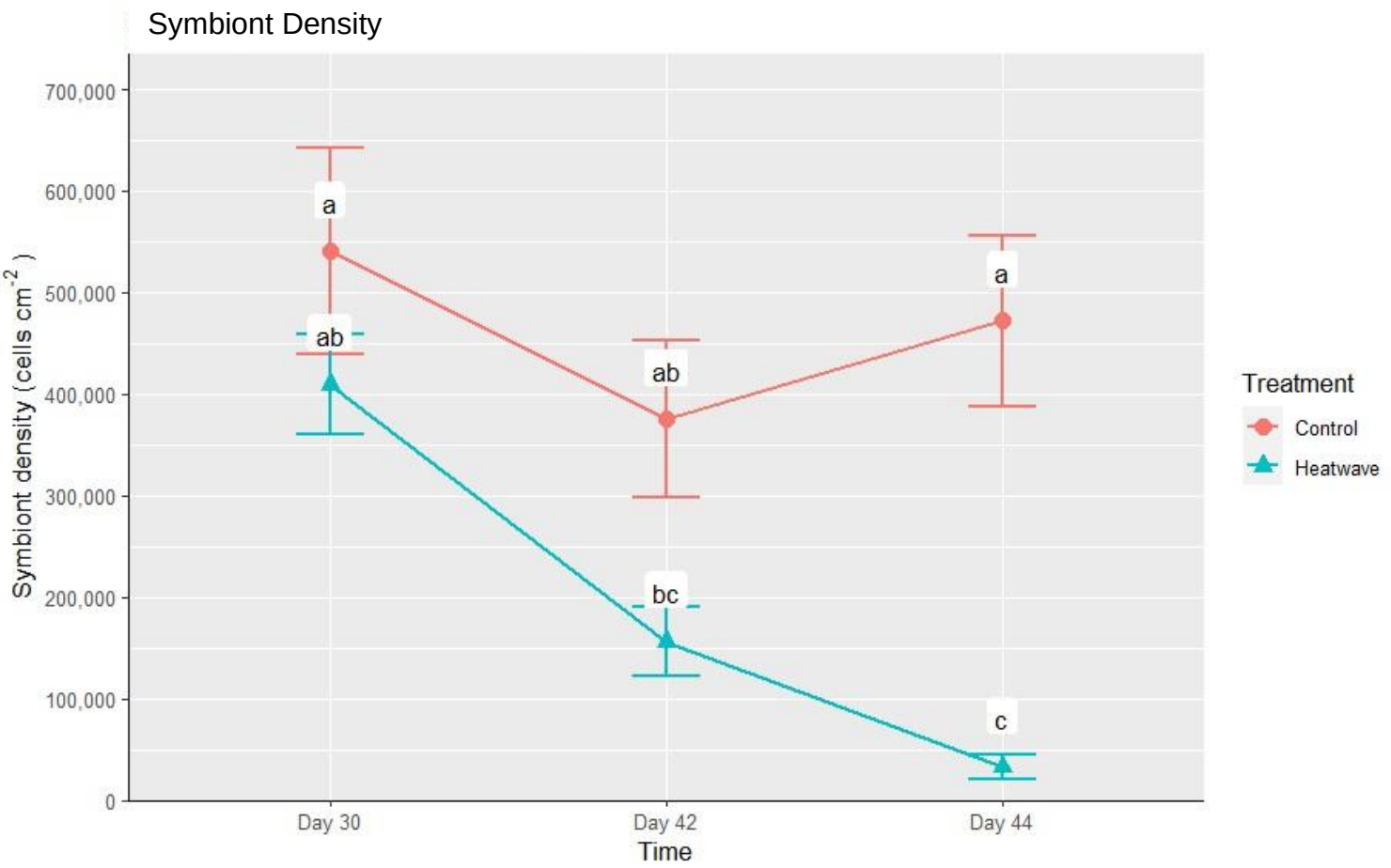


Fig. 4. Symbiont density in cells per square centimetre of the control (red circles) and heatwave treatment (blue triangles) at three moments during the experiment. Treatments with the same letters are not significantly different. Error bars indicate the standard error.

The two-way mixed ANOVA showed a significant main effect of both heat treatment ($F = 23.129$, $df = 1$, $p < 0.001$) and time ($F = 6.948$, $df = 2$, $p < 0.01$) (Appendix 3.2). Furthermore, multiple comparison tests were conducted to identify which treatment and measurements differed significantly (Appendix 3.3). These multiple comparison tests resulted in six significant differences (Table 1).

Table 1. Significant results of the multiple comparison tests, including the decline in symbiont density.

| Comparison | P-value | Decline in symbiont density |
|---|---|---|
| Control Day 30 – Heatwave Day 42 | 0.0082 | 71% |
| Control Day 30 – Heatwave Day 44 | 0.0005 | 94% |
| Control Day 42 – Heatwave Day 44 | 0.0206 | 91% |
| Control Day 44 – Heatwave Day 42 | 0.0367 | 67% |
| Control Day 44 – Heatwave Day 44 | 0.0024 | 93% |
| Heatwave Day 30 – Heatwave Day 44 | 0.0097 | 92% |

### *3.2.2. Recovery of symbiont density*

On day 69, 29 days after the bleaching started during the recovery, another symbiont count was conducted. Only two corals from the control and heatwave treatment were used this time instead of the usual four because these corals were sacrificed for the *Symbiodiniaceae* sequencing, and later, the decision was made that these pellets could also be used to conduct a symbiont count. The results showed that the average symbiont density in the heatwave corals was 4,161 cells per $cm^2$ compared to the 158,102 cells per $cm^2$ in the control corals, resulting in a difference of 97.4%. This difference between the control and heatwave treatment was significant (t-test; $t = 4.303$, $df = 2$, $p = 0.004$).

## *3.3. Frequency of dividing symbionts*

The frequency of dividing symbionts in the heatwave corals was counted during the symbiont counts on day 42 (three days after bleaching), day 44 (five days after bleaching), and day 69 (recovery/29 days after the bleaching started). The frequency of dividing symbionts on days 42 and 44 was 3%. The counts of dividing symbionts were higher on day 42 than on day 44, but due to the higher symbiont density on day 42, they both had 3%. On day 69, during the recovery, this percentage was 8%, so higher than during the bleaching. However, only two corals were used during this last count instead of four for the other two measurements, where one coral had 17% dividing cells, whereas the other had none. To put it into perspective, the control corals had an average of 5% of dividing symbionts.

## *3.4. Green fluorescent protein (GFP)*

GFP was measured on days 42 and 44 (Fig. 5) during the bleaching. The two-way mixed ANOVA showed a significant decrease over time ($F = 8.371$, $df = 1$, $p < 0.01$) (Appendix 3.4). Out of the multiple comparison tests, only the GFP between the control treatment on day 44 and the heatwave treatment on day 42 came out significant ($p < 0.05$) (Appendix 3.5). Compared to the control treatment, the GFP of the heatwave treatment increased by 11% on day 42 and 32% on day 44. Visually, the corals in the heatwave treatment first became more fluorescent yellow around day 40 and within a couple of days, the corals had turned pale and white.

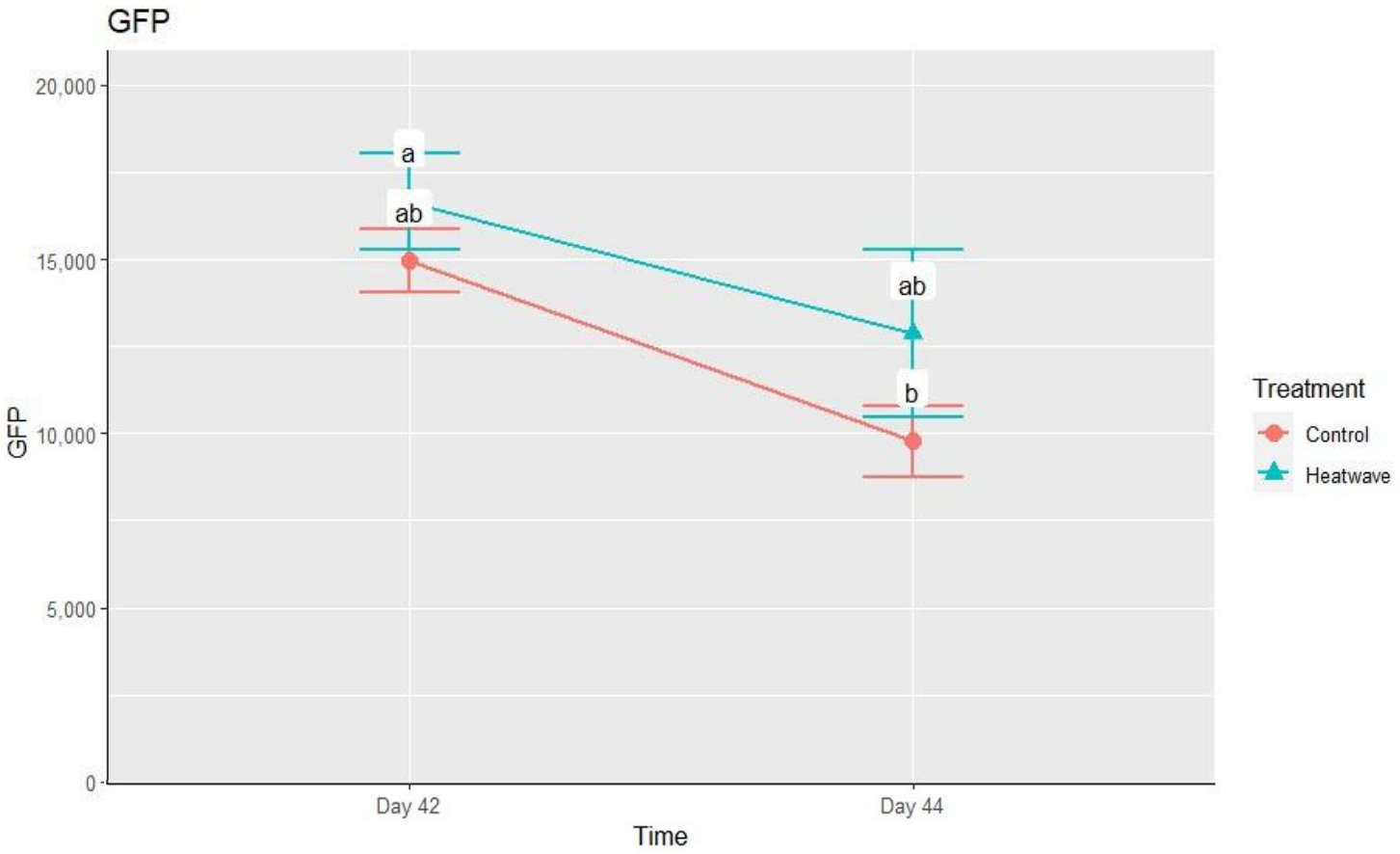


Fig. 5. GFP (in arbitrary units, normalised per $cm^2$ of coral surface) of the control (red circles) and heatwave treatment (blue triangles) on day 42 and 44. Treatments with the same letters are not significantly different. Error bars indicate the standard error.

### *3.5. Symbiodiniaceae sequencing*

Three samples were sequenced based on the best PCR results. Two of the three samples had a good sequence result. It was able to identify with 99.24% certainty that the most dominant symbiont type inside *Caulastrea furcata* is *Cladocopium sp.* However, the outcome was not specific enough to identify the exact species of *Cladocopium*. Still, the most dominant symbiont type inside the Caulastrea furcata corals used for this experiment is now known. The results of the DNA isolation and PCR are presented in Appendix 4.

### *3.6. Temperature, salinity & water quality*

The monitoring data of the temperature, salinity, and water quality (alkalinity, calcium, phosphate, and nitrate) during the experiment are presented in figures in Appendix 5 (temperature & salinity) and Appendix 6 (water quality).

# 4. Discussion

This study aimed to gain knowledge on the response of Indo-Pacific corals to long exposure to a presumed sublethal temperature. In addition, I wanted to distinguish what exactly happens with the symbiosis between the coral host and the symbionts under prolonged exposure to heat stress, so if Type I or Type II bleaching would occur.

The major finding of this study is that the Indo-Pacific coral *Caulastrea furcata* bleached at 31°C. This was according to what Sam Nietzer of the University of Oldenburg expected. However, what was unexpected was that bleaching coincided with a crash in effective yield (Type I bleaching). So, the hypothesis that corals bleach at 31°C because of a disrupted energy balance by a release of healthy symbionts (Type II bleaching) can be rejected. In conclusion, the bleaching response of *Caulastrea furcata* after long exposure to a presumed sublethal temperature was that the symbiont photosystem was negatively affected rather than the coral-algal symbiosis.

Additional findings include a decline of the symbiont density during the bleaching process. Regarding the first sub-question, whether the symbionts get either expelled or killed due to heat stress, no clear evidence was found that the symbionts got expelled during the bleaching. Considering that the effective yield crashed and the symbiont density was reduced, the symbionts were most likely killed due to heat stress. However, the frequency of dead symbionts was not monitored, so this cannot be confirmed.

The final finding was that the most dominant symbiont type inside the *Caulastrea furcata* corals used for this experiment was *Cladocopium sp*. This dominant symbiont type was likely related to the bleaching response. In the following sections, I explain the results in more detail while putting it in a broader perspective.

Temperature-induced photosynthetic impairment was observed after eight days at 31°C. The effective yield declined within the next five days by 89%. Pulse-amplitude-modulated (PAM) fluorometry has been used to measure the effective yield for over 20 years (Freeman et al., 2001; Jones et al., 1999; Ralph et al., 2001). Generally, the value of a healthy coral sits between 0.6 and 0.7 (Okamoto et al., 2005), and when this value drops below 0.5, the coral starts to get paler. As a rule of thumb, this is when the bleaching starts (Okamoto et al., 2005). This trend can also be observed in the results of this study. Until day 40, the effective yield value of the heatwave and the control corals averaged around 0.6. This is inside the healthy margin. There might be several explanations for the effective yield dropping like this. However, based on the effective yield and symbiont density results, the most likely explanation is the dysfunction of the symbionts in the photosystem due to the sublethal heat stress (Warner et al., 1999). Both the corals and symbionts were still alive, but the number of symbionts and their effective yield declined.

After the bleaching, the effective yield showed no recovery. Four effective yield measurements were conducted, each one week apart. Unfortunately, the first three measurements were all zero. The fourth and last measurement had a slight increase to 0.239. However, this was due to a change in the sensitivity of the MINI-PAM-II rather than a biological process. This last value was still too low to suggest recovery. This indicated that the photosystem of the symbionts was heavily damaged due to the heat stress. A possible explanation for the lack of recovery might be the temperature ramping. I increased the temperature by 1°C every four days to simulate a natural bleaching event. However, this is still extremely fast compared to what happens in nature. In nature, the sea surface temperature increases more gradually. This might have damaged the photosystem even more. Recovery might still be possible because there are still a few symbionts inside the corals, but it might take longer. In nature, recovery usually takes place between 4 and 6 weeks (Allen-Waller & Barott, 2023), and for this study, I monitored recovery for only four weeks due to lack of time. Monitoring is still ongoing at the time of writing but is not included in this report.

The decreased symbiont density was also a clear sign that the corals were bleaching. Within two days, the number of symbionts declined by 90%. Nevertheless, still 30,000 symbionts per $cm^2$ were counted after bleaching. So, in total, many symbionts were still inside the corals during the bleaching process. Moreover, during the recovery period, a month after the bleaching started, more than 4,000 cells per $cm^2$ were still inside the corals. This seems low compared to the 400,000 cells per cm2 during the temperature ramping. However, in total, there are still a lot of symbionts present. For the symbiont density measurements during the recovery, only two heatwave corals were used instead of the usual four, so this data cannot be statistically compared with the other measurements. Only two heatwave corals were used for this measurement because these corals were sacrificed for the *Symbiodiniaceae* sequencing. So, the decision was later made to use the pellets of these corals to conduct a symbiont density and frequency of dividing symbiont count.

Concerning the frequency of dividing symbionts, the measurements during the bleaching were similar to the average percentage measured in the control corals. However, something interesting was observed in the symbiont count during the recovery. One coral showed a frequency of dividing symbionts of 17%, whereas in the other heatwave coral, no dividing symbionts were observed. Similar to the symbiont density, the recovery measurement cannot be statistically compared to the measurements during the bleaching because two corals were used instead of four for the same reason as above. Because of this high frequency of dividing symbionts in one of the heatwave corals, the average dividing symbionts was the highest during the recovery. The high percentage of dividing cells in one of the corals shows a sign of recovery. There is no apparent reason to believe this high number is a mistake. The low percentage of dividing symbionts before the recovery renders it unlikely that the symbionts were putting more energy into cell division due to ammonium production at this stage. This again suggests that the hypothesis of the dysfunction of the symbiosis between the coral host and the symbionts causing bleaching (Rädecker et al., 2021) can be debunked. Consequently, the photosystem of the symbionts was damaged due to the heat stress, supporting the hypothesis of oxidative stress being the cause of bleaching.

Green fluorescent protein (GFP) concentration and fluorescence decrease with declining coral health before bleaching (Roth & Deheyn, 2013). In this experiment, GFP was only measured during bleaching, and the values for the control and heatwave corals were similar. However, it showed a slight decrease in both control and heatwave corals. Before the bleaching, the heatwave corals were visually more fluorescent than the control corals. Also, during the bleaching, this was still the case. Roth & Deheyn (2013) found that the bleached heat-treated corals showed strong fluorescence despite reduced GFP concentration. The results of this experiment are in line with this finding. The study explained that the GFPs become more visible due to reduced shading caused by the decreased symbiont density inside the bleached corals. However, it cannot be confirmed whether the cause of the decrease in symbiont density was expulsion or death.

*Cladocopium sp.* was the most dominant symbiont type inside the corals used for this experiment. The University of Oldenburg, which provided the corals, has a connection to Guam, so these corals originated from there. *Cladocopium* occurs widely in the Indo-Pacific, from temperate Japan (Lien et al., 2012) to the Great Barrier Reef (Beltrán et al., 2021). Therefore, this genus is adapted to a wide temperature range, including heat and cold tolerance. A possible explanation of the bleaching response observed during this experiment was that *Cladocopium* acts the same as *Symbiodinium*, the genus present in corals used in past experiments and studies at Wageningen University & Research, but at a lower temperature. However, the specific species of *Cladocopium* is unknown, so I cannot confirm whether this is the case. The PCR results of the three samples were good, so it is not likely that that was why it did not give a clear answer. A possible explanation could be that no specific symbiont species was the most dominant during the bleaching. Therefore, it was unclear which specific species was present at that time. So, it might have been a combination of multiple symbiont species. Maybe this is an indication of symbiont shuffling.

Recommendations and future perspectives include studying the potential kicking out of healthy symbionts by the coral host. The timing of measurement must be perfect for studying the expelling of symbionts. So, you have to be able to determine precisely when the bleaching process exactly starts. Therefore, the bleaching threshold must be known. A smaller volume of the experiment tanks decreases the dilution of the symbionts when they are expelled. So, the collection of the symbionts might be easier. In addition, the pumps should then be turned off immediately to prevent the expelled symbionts from washing away by the current and to collect water samples for further analysis. If evidence for this process can be found, it can be used to refine and test the hypothesis that the dysfunction of the symbiosis between the coral host and the symbionts triggers coral bleaching.

Another recommendation is the identification of the symbionts at the species level. As the underlying process is species-specific, this would clarify whether shifts to more resilient symbiont species occur during and after bleaching.

Finally, placing *Cladocopium* inside different coral hosts could provide valuable insights into the differences in bleaching responses to study the exact role *Cladocopium* has on the bleaching response of its host. To put it into a broader perspective, exchanging *Symbiodiniaceae* genera between coral hosts might be a high-potential bleaching mitigation measure.

# 5. Conclusions

In conclusion, the bleaching threshold of *Caulastrea furcata* is lower than the threshold for corals, which were used in past experiments and studies in Wageningen. The corals started to bleach after eight days at 31°C in this experiment.

To answer the main question, it is most likely that the bleaching response of *Caulastrea furcata* to long exposure to the presumed sublethal temperatures stress affected the symbiont photosystem rather than the coral-algal symbiosis. A potential imbalance of nutrients was not the reason that no increase in the cell division rate of the symbionts was observed during the bleaching. In addition, the symbiont density declined significantly during the bleaching. The decline in symbiont density and the effective yield suggest that the symbionts get killed when exposed to heat stress. Furthermore, no clear evidence of symbiont expulsion was found. However, the frequency of dead symbionts was not monitored during the experiment, so this cannot be confirmed.

Symbiont sequencing showed that the dominant symbiont type in this specimen of *Caulastrea furcata* is *Cladocopium sp*. This symbiont type is widely distributed across the Indo-Pacific from temperature Japan to the Great Barrier Reef, meaning that this genus is adapted to a wide temperature range, including heat and cold tolerance. The corals for this experiment originated from Guam in the Indo-Pacific, compared to those used in past experiments and studies in Wageningen, which came from the Red Sea. *Caulastrea furcata* originates from a relatively colder region. So, although *Cladocopium* shows the same bleaching reactions as *Symbiodinium*, they occur at a slightly lower temperature.

# 6. Acknowledgements

I would like to thank my supervisors, Ronald Osinga and Pim van Hooft, for their help and support during my MSc thesis. Also, I want to thank Diederik Padmos for his help in the lab and Britt van Herpen for her assistance in the *Symbiodiniaceae* sequencing. Furthermore, I want to thank the Marine Animal Ecology chair group, especially Diede Maas, for the thesis ring meetings and the general support. Also, I would like to thank Sam Nietzer from the University of Oldenburg for kindly providing the corals. Lastly, I would like to thank Mirjam Cevat, my study advisor, and Martijn van der Spoel, my WUR psychologist, for their support during this period.

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# Appendices

## *Appendix 1: Protocol Symbiont Density Measurement*

*Removing the tissue from a piece of coral*

1. Fill the water pick reservoir with clean seawater. Put it on floss mode and use flow velocity 5 for the best result.
2. Use parafilm to cover a 500 mL beaker glass and poke two holes for the tweezer and the waterpik.
3. Hold the piece of coral inside the 500 mL beaker glass with a tweezer. Rinse the tissue of the coral skeleton with the water pick. Put your safety glasses on while doing this to prevent chunks of coral tissue (which may contain sting cells) from ending up in your eyes! You will need at least 50 mL of tissue suspension for further processing. Try to limit excessive use of water, because this will dilute the cell suspension too much.
4. Bring the obtained tissue suspension in the beaker glass over into a 100 mL graded cylinder. Rinse the empty beaker glass with the water pick with a few mL of seawater and add this volume to the tissue suspension in the graded cylinder. Note down the total volume obtained in the cylinder. We call this "Volume 1".
5. Fill a 50 mL Falcon tube up to exactly 50 mL with the tissue suspension from the graded cylinder.
6. Centrifuge for 15 minutes at 5000 rpm.

*Determination of the coral surface*

1. Measure the length of the coral piece (L in Fig. 6)
2. Measure the radius of the coral piece (r in Fig. 6)
3. Calculate the coral surface (A) by assuming a cylindrical shape: A = 2*π*Radius*L. Convert to cm2 as the final unit.

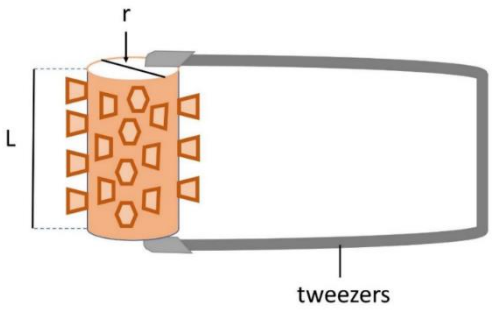



Figure 6. Coral sample held with tweezer. Radius = r /2 and the length is L.

*Determination of the symbiont density*

1. After centrifugation, decant the supernatant from the 50 mL tube
2. Resuspend the pellet with 750 uL clean seawater (use pipet with blue tips)
3. After homogenisation, determine the volume of the new suspension by adjusting the pipet volume until it takes up exactly the entire suspension (we call this "Volume 2")
4. Divide a subsample of 20 uL of Volume 2 over the two sides of a Neubauer Improved counting chamber. Put the chamber under a light microscope, using 10 x 10 magnification. Count all algae cells in one square of 1 x 1 mm on both sides of the chamber. Do not count symbionts which are situated on the border of the 1 x 1 mm grid (Fig. 7).

Calculate the density in cells cm2. First, multiply the average cell count by 10 to obtain the cell concentration in the centrifuged sample. Then, divide Volume 2 by the volume in the centrifuge tube and multiply it by the cell concentration in the centrifuged sample. Now use this concentration to calculate the amount of symbionts present by multiplying it with Volume 1 * 1000. Calculate the symbiont density by dividing the total amount of symbionts by the surface area.

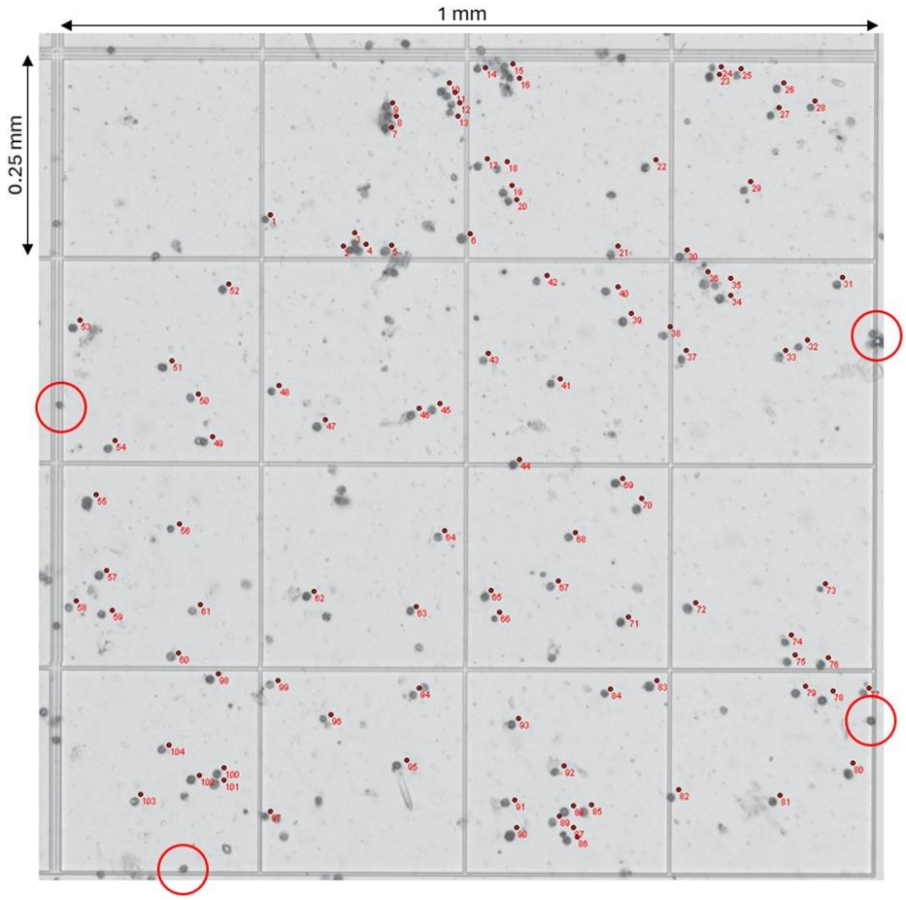



Fig. 7. 1 x 1 mm grid

# *Appendix 2: Protocol Symbiodiniaceae Sequencing*

**General remarks for molecular analysis:**

For small volumes (<5 µL), use the Tick & spin (T&S) technique to make sure all of the sample is mixed and at the bottom of the eppje.

Make sure you place samples in Lobind eppjes

Don't forget to add negative controls!!: Negative DNA control (during DNA extraction) and negative PCR control

**Nanodrop**

1) Clean the Nanodrop with a paper tissue
2) First add 1 µL C6 from Power Soil Pro Kit to measure BLANK
3) Clean Nanodrop when switching between samples
4) Use the T&S and add 1 µL of a sample to the Nanodrop and click 'Measure'
5) Write down the value of ng/µL
6) Repeat steps 3-5 for all samples

Write nanodrop results as:

| Date of extraction | Sample | ng/µL | A260 | 260/230 | 260/280 | Graph shape |
|---|---|---|---|---|---|---|
| *24/5/23* | *SPG* | *19,686* | *0,3937* | *0,351* | *1,938* | |

**PCR**

**Tips for PCR MIX from Metabarcoding course:**

- Try to pipette up & down for almost all things to ensure it is mixed before using it
- Wait for a moment when pipetting mastermix, due to viscosity a drop can remain within the pipette tip.
- Make sure your primers have already been diluted!!

| Primer pair | Sequence 5′–3′[1] | Amplicon size bp | Protocol[2,3] | Cycles[1] | Publication |
|---|---|---|---|---|---|
| SYM_VAR_5.8S2[2] | GAATTGCAGAACTCCGTGAACC | ~234–266 | 98 °C for 2 min | 35 | Hume et al. (2015) |
| SYM_VAR_REV[2] | CGGGTTCWCTTGTYTGACTTCATGC | | 98 °C for 10 s, 56 °C for 30 s, 72 °C for 30 s 72 °C for 5 min | | Hume et al. (2013) |

**Mix for PCR:**
=> for 24 samples (21 samples + negative control + 10-20% extra for pipetting error)

| "Ingredient" | Proportion (µL) | For 24 samples (µL) |
|---|---|---|
| • Primer Forward SYM_VAR_5.8S2 | 0,1 | 2,4 |
| • Primer Reverse SYM_VAR_REV | 0,1 | 2,4 |
| • Master Mix (phire) | 5 | 120 |
| • Mili Q water (MQ) | 5,8 | 139,2 |
| | Total volume: 11 µL | 264 µL |

> Mix together in 1 eppje. Then add 11 µL to PCR strip within ice block container (purple)

- T&S and add 1 µL of each template extracted DNA sample to the PCR strip and write down (!Important that this is done last!)
- **Tips for PCR strip from Metabarcoding course:**
  - Never go further than treshold of pipette and up again within the pcr strip, as a small volume is lost within the tip which cannot be retrieved
  - Try to open pcr strip without creating too much current, by opening it all at once, not from left to right.
  - Normally, PCR is done in triplo, because PCR can have a bias

Mark strip in labjournal, like:

| | | | |
|---|---|---|---|
| A | SF3 | E | SF13 |
| B | SF5 | F | SF14 |
| C | SF8 | G | Water / negative control |
| D | SF11 | H | Water / negative control |

**PCR Temperatures & Times**

- T&S PCR strip before placing it in the PCR machine
- Set the volume to 12 µL (11 µL Mastermix + 1 µL DNA)

| Temperature | Time |
|---|---|
| 98 | 2 min |
| 98 | 10 s \ |
| 56 | 20 s --> repeat 34x |
| 72 | 20 s / |
| 72 | 5 min (or infinite) |

**Making Gel**

- 100 mL TBE buffer
- 1,7 % agarose

1) Add ~50-70 mL TBE + 1,7 g agarose to erlenmeyer and heat till boiling + all is dissolved
2) Add remainder of TBE to erlenmeyer
3) Check whether erlenmeyer is cool to the touch, otherwise cool some more under cold water
4) Add 1 µL Stain G
5) Check whether MasterMix makes the DNA heavier (to let it sink to the bottom)! Otherwise add Loading buffer (-> Phire has loading buffer in it, so we can skip this step)
6) Pour the mix into the mold and include the appropriate comb
7) It takes approx. 20 min to cool down

**Adding PCR products to gel**

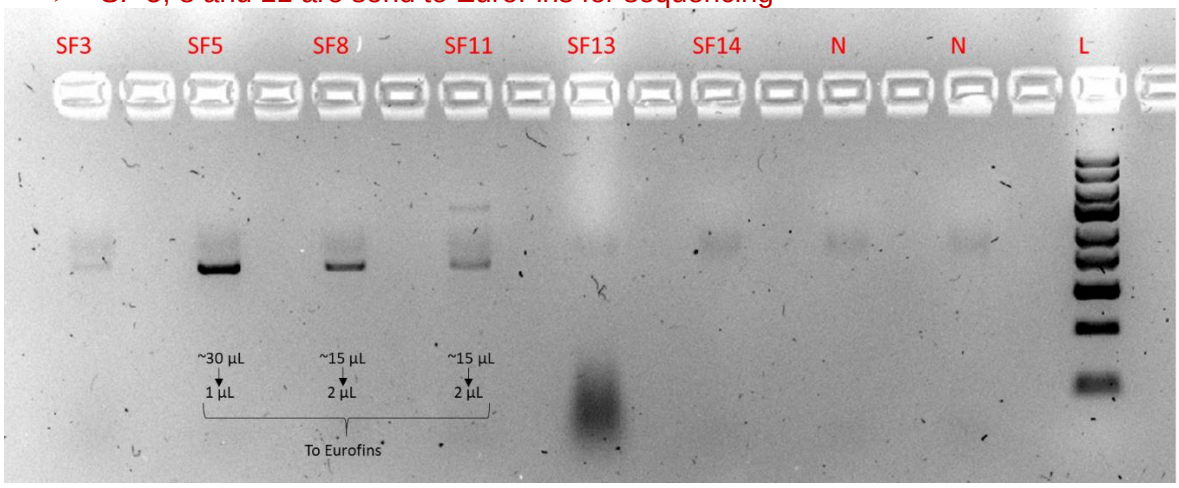
1) Place gel in bath (just under enough TBE)
2) Place 2 µL of PCR products in the wells
3) Also add a ladder! Check for which baselength the ladder is sufficient
4) Set at 120 V for 40 min

**Gel imaging**

- Place gel (not the mold) in the machine)
- Program = ...
- Select New Protocol
- > Nucleic Acids > Ethidium Bromide
- Position gel: filter 1
- Place wells on line
- Run
- To save: Export > for analysis > JPEG
- Results:

Example of possible results:
Good bands: SF 5 & 8 (both lobster samples)
SF11 had 2 bands, so not that good
SF 3 and SF 13 (both brittle stars) showed smears, probably because they had a too high DNA concentration --> next time dilute the samples for PCR analysis
SF 14 had too low DNA concentration to show a band
Negative DNA and PCR control has no band (=good!)

- SF 5, 8 and 11 are send to EuroFins for sequencing




**Sanger sequencing sending:**

Includes:

- 15 µL nuclease free water
- 2 µL Forward Primer (SYM_VAR_5.8S2)
- ... µL PCR product with end concentration ~ 30 ng/ µL
  (1 µL for SF5, 2 µL for SF8 and SF11)

| *Barcode* | *Sample* | *Code* | µL PCR |
|---|---|---|---|
| *EF71815034* | *4* | *C4 28-5* | *2* |
| *EF71815035* | *5* | *H1 28-5* | *2* |
| *EF71815036* | *19* | *H1 24-6* | *1* |

# *Appendix 3: Results*

## *3.1. Effective yield t-test data*

Table 2. Effective yield

| Day | T-value | Degrees of freedom | P-value |
|---|---|---|---|
| 40 | 1.993 | 62 | 0.01 |
| 41 | 8.291 | 62 | 2.07e-12 |
| 42 | 4.245 | 50 | 1.56e-05 |
| 43 | 8.782 | 37 | 2.12e-11 |
| 44 | 12.578 | 59 | 3.67e-17 |
| 45 | 12.578 | 59 | 3.67e-17 |

## *3.2. Symbiont density ANOVA*

```
               Df    Sum Sq   Mean Sq F value   Pr(>F)
Time            2 2.494e+11 1.247e+11   6.948 0.005805 **
Treatment       1 4.150e+11 4.150e+11  23.129 0.000141 ***
Time:Treatment  2 1.010e+11 5.050e+10   2.814 0.086394 .
Residuals      18 3.230e+11 1.794e+10
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Fig. 8. Symbiont density ANOVA

## *3.3. Symbiont density multiple comparison test data*

```
Comparison between treatments means

                                                 difference pvalue signif.       LCL      UCL
Control at Day 30 - Control at Day 42             165137.37 0.5230         -135886.99 466161.7
Control at Day 30 - Control at Day 44              68345.53 0.9767         -232678.84 369369.9
Control at Day 30 - Heatwave at Day 30            130770.51 0.7375         -170253.85 431794.9
Control at Day 30 - Heatwave at Day 42            384089.47 0.0082       **   83065.10 685113.8
Control at Day 30 - Heatwave at Day 44            507629.28 0.0005      ***  206604.92 808653.6
Control at Day 42 - Control at Day 44             -96791.84 0.9045         -397816.21 204232.5
Control at Day 42 - Heatwave at Day 30            -34366.86 0.9990         -335391.22 266657.5
Control at Day 42 - Heatwave at Day 42            218952.10 0.2397          -82072.26 519976.5
Control at Day 42 - Heatwave at Day 44            342491.91 0.0206        *   41467.55 643516.3
Control at Day 44 - Heatwave at Day 30             62424.98 0.9843         -238599.38 363449.3
Control at Day 44 - Heatwave at Day 42            315743.94 0.0367        *   14719.58 616768.3
Control at Day 44 - Heatwave at Day 44            439283.75 0.0024       **  138259.39 740308.1
Heatwave at Day 30 - Heatwave at Day 42           253318.96 0.1296          -47705.40 554343.3
Heatwave at Day 30 - Heatwave at Day 44           376858.77 0.0097       **   75834.41 677883.1
Heatwave at Day 42 - Heatwave at Day 44           123539.81 0.7791         -177484.55 424564.2
```

Fig. 9. Symbiont density multiple comparison

```
Treatments with the same letter are not significantly different.

                  symbionts groups
Control at Day 30  540413.46      a
Control at Day 44  472067.94      a
Heatwave at Day 30 409642.95     ab
Control at Day 42  375276.09     ab
Heatwave at Day 42 156323.99     bc
Heatwave at Day 44  32784.18      c
```

Fig. 10. Symbiont density multiple comparison

### *3.4. GFP ANOVA*

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|---|
| Time | 1 | 160593150 | 160593150 | 8.371 | 0.0073 | ** |
| Treatment | 1 | 45788369 | 45788369 | 2.387 | 0.1336 | |
| Time:Treatment | 1 | 3810689 | 3810689 | 0.199 | 0.6593 | |
| Residuals | 28 | 537158148 | 19184220 | | | |

---

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Fig. 11. GFP ANOVA

### 3.5. *GFP multiple comparison test data*

Critical Value of Studentized Range: 3.861244

Comparison between treatments means

| | difference | pvalue | signif. | LCL | UCL |
|---|---|---|---|---|---|
| Control at Day 42 - Control at Day 44 | 5170.589 | 0.1084 | | -808.7653 | 11149.943 |
| Control at Day 42 - Heatwave at Day 42 | -1702.222 | 0.8641 | | -7681.5765 | 4277.132 |
| Control at Day 42 - Heatwave at Day 44 | 2088.024 | 0.7765 | | -3891.3298 | 8067.379 |
| Control at Day 44 - Heatwave at Day 42 | -6872.811 | 0.0196 | * | -12852.1654 | -893.457 |
| Control at Day 44 - Heatwave at Day 44 | -3082.564 | 0.5053 | | -9061.9187 | 2896.790 |
| Heatwave at Day 42 - Heatwave at Day 44 | 3790.247 | 0.3274 | | -2189.1075 | 9769.601 |

Fig. 12. GFP multiple comparison

## *Appendix 4: DNA Isolation & PCR Results*

Table 3. DNA isolation

| Date of extraction | Sample nr. | Sample code | ng/µL | A260 | 260/230 | 260/280 |
|---|---|---|---|---|---|---|
| 25-Jun | 1 | C1 28-5 | 10.413 | 0.2083 | 0.987 | 1.985 |
| 25-Jun | 2 | C2 28-5 | 12.744 | 0.2549 | 0.311 | 2.007 |
| 25-Jun | 3 | C3 28-5 | 4.931 | 0.0986 | 0.448 | 1.731 |
| 25-Jun | **4** | **C4 28-5** | 9.662 | 0.1932 | 0.227 | 1.828 |
| 25-Jun | **5** | **H1 28-5** | 15.769 | 0.3154 | 0.985 | 1.874 |
| 25-Jun | 6 | H2 28-5 | 8.363 | 0.1673 | 0.324 | 1.829 |
| 25-Jun | 7 | H3 28-5 | 16.965 | 0.3393 | 0.566 | 1.902 |
| 25-Jun | 8 | H4 28-5 | 10.474 | 0.2095 | 1.512 | 1.901 |
| 25-Jun | 9* | C1 30-5 | 7.602 | 0.152 | 0.357 | 1.679 |
| 25-Jun | 10 | C2 30-5 | 12.918 | 0.2584 | 0.467 | 1.922 |
| 25-Jun | 11 | C3 30-5 | 9.339 | 0.1868 | 0.437 | 1.668 |
| 25-Jun | 12 | C4 30-5 | 6.951 | 0.139 | 1.254 | 1.698 |
| 25-Jun | 13 | H1 30-5 | 9.347 | 0.1869 | 0.118 | 1.854 |
| 25-Jun | 14* | H2 30-5 | 8.241 | 0.1648 | 0.191 | 1.796 |
| 25-Jun | 15* | H3 30-5 | 2.806 | 0.0561 | 0.324 | 1.803 |
| 25-Jun | 16 | H4 30-5 | 7.617 | 0.1523 | 0.07 | 1.659 |
| 25-Jun | 17* | C1 24-6 | 1.03 | 0.0206 | 0.015 | 0.983 |
| 25-Jun | 18 | C2 24-6 | 13.741 | 0.2748 | 1.294 | 2.012 |
| 25-Jun | **19 *** | **H1 24-6** | 41.772 | 0.8354 | 1.674 | 1.958 |
| 25-Jun | 20* | H2 24-6 | 38.687 | 0.7737 | 2.323 | 1.887 |
| 25-Jun | 21 | BLANK | 1.669 | 0.0334 | 0.085 | 1.324 |

* Whole pellet used instead of half

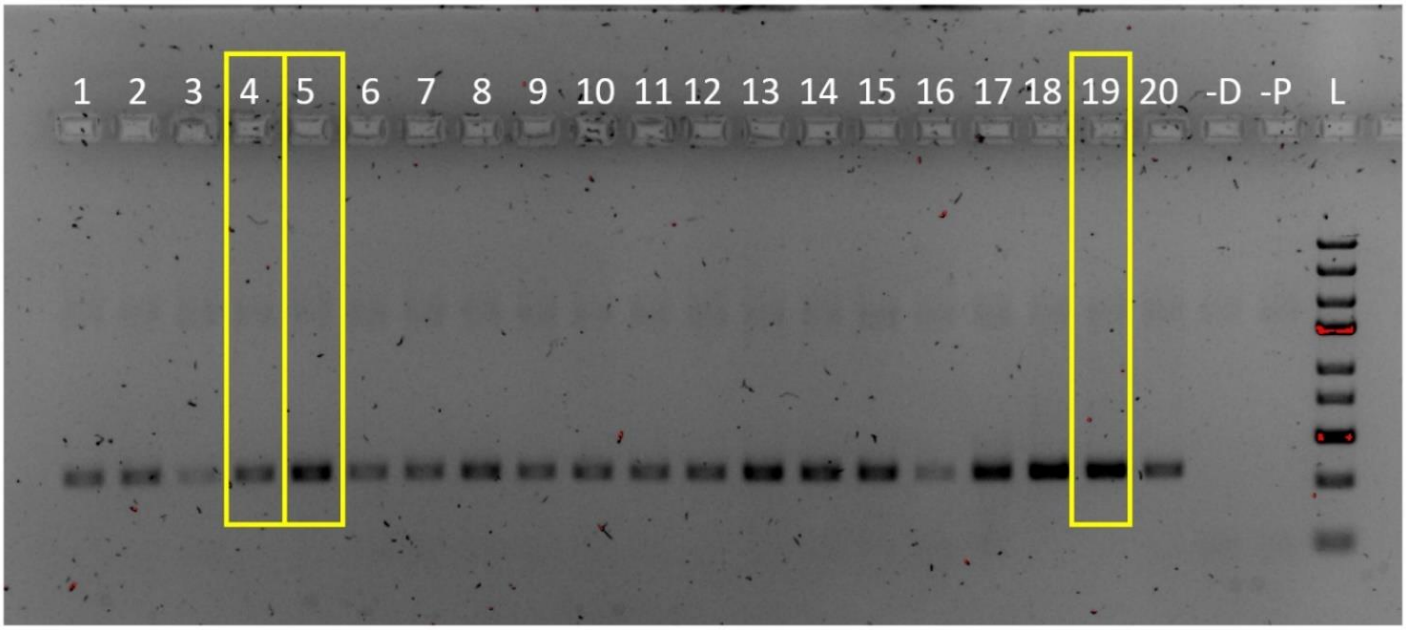



Fig. 13. PCR result

## *Appendix 5: Temperature & Salinity*

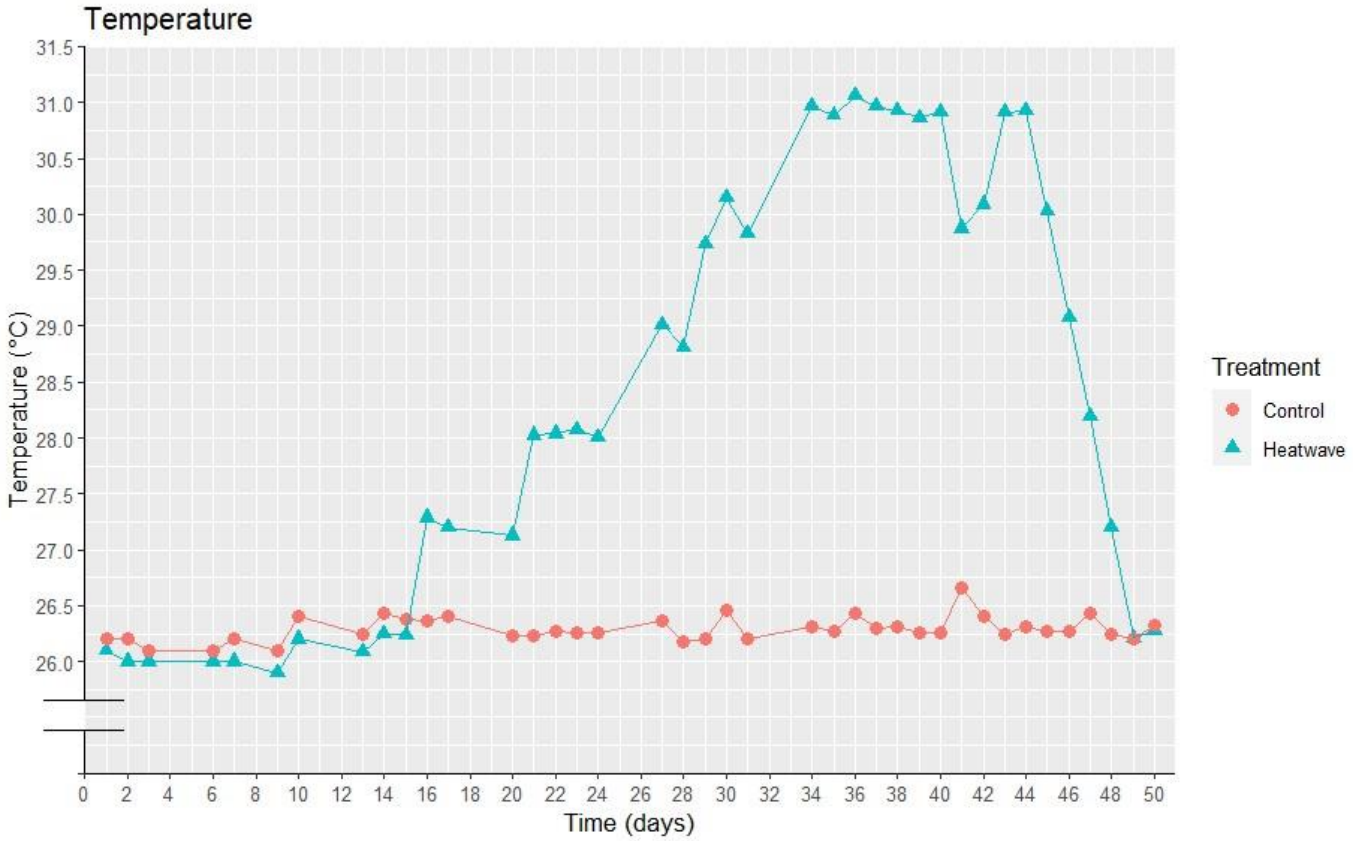



Fig. 14. Temperature

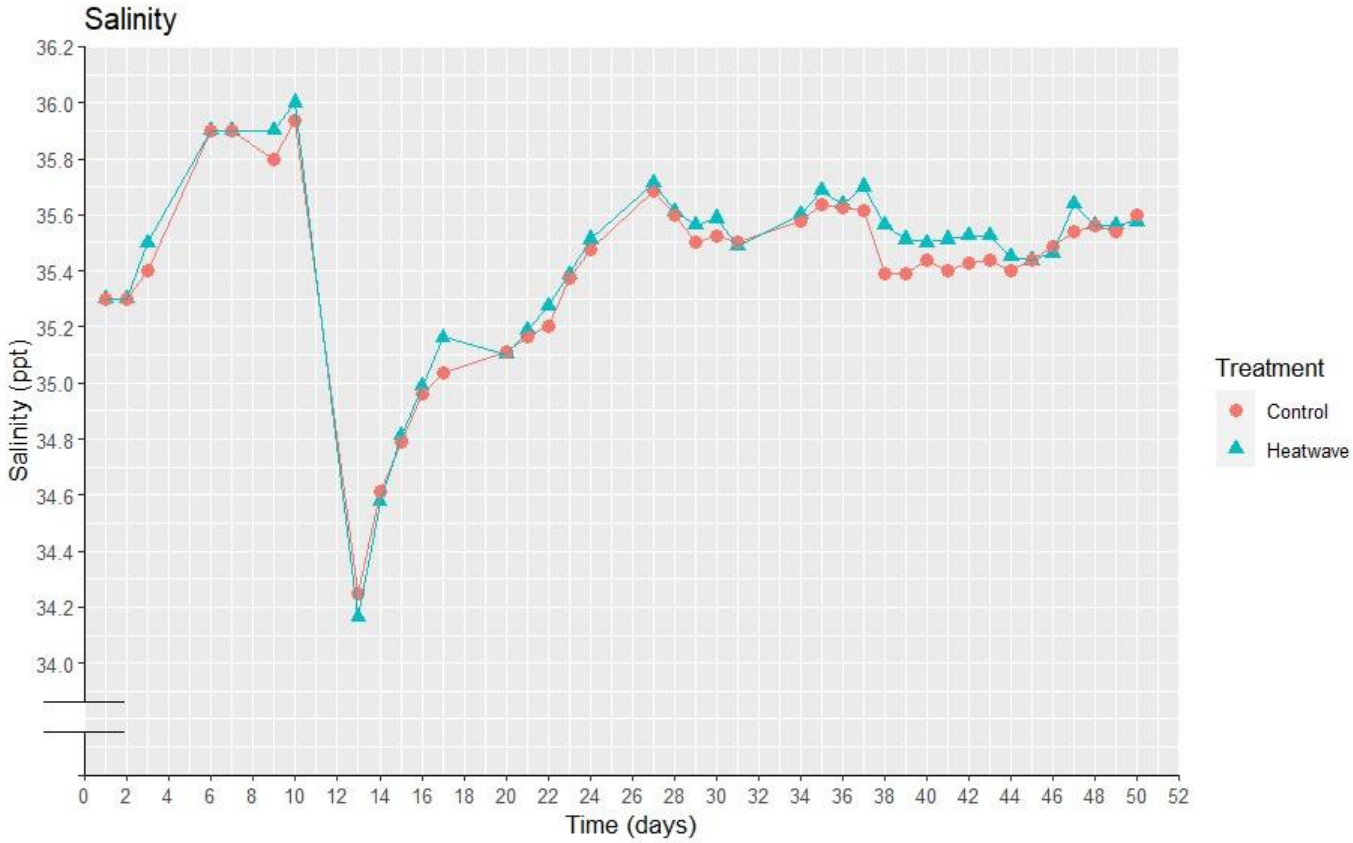



Fig. 15. Salinity

## *Appendix 6: Alkalinity, Calcium, Phosphate & Nitrate*

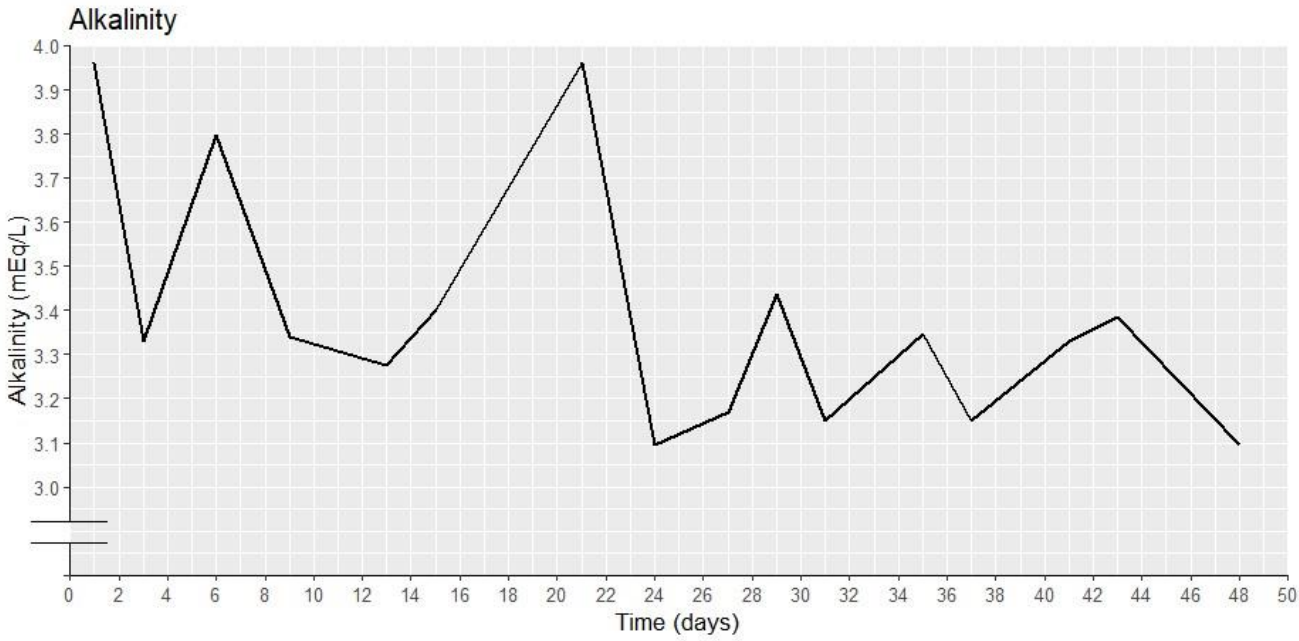



Fig. 16. Alkalinity

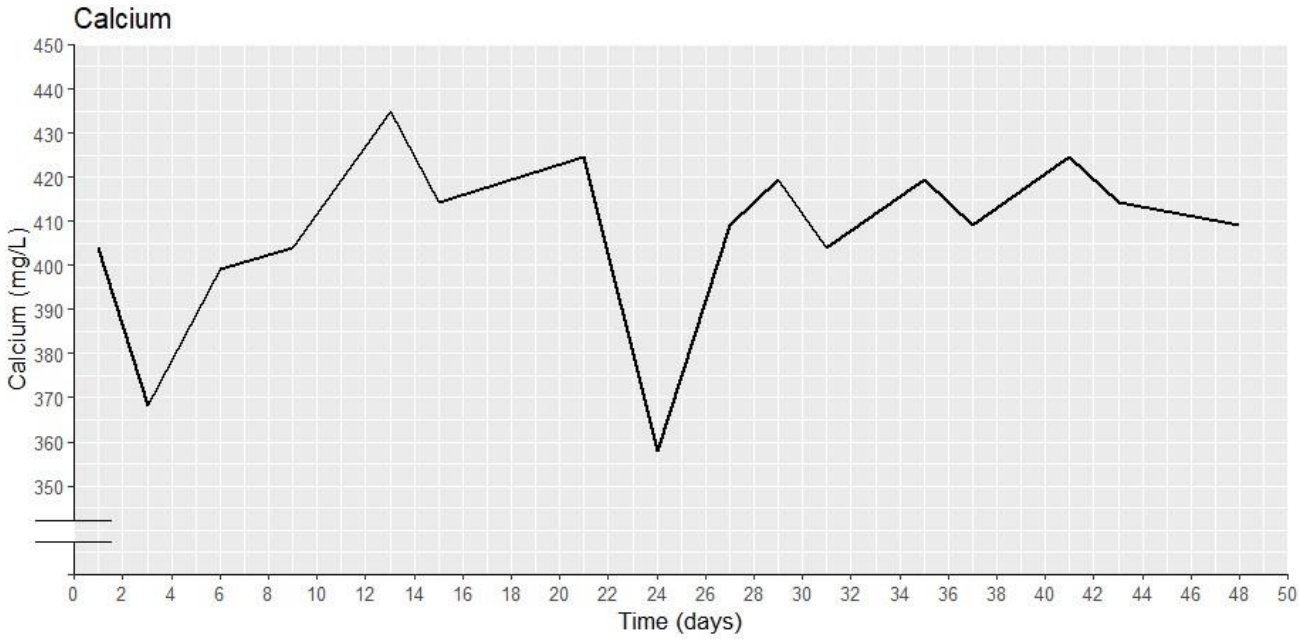



Fig. 17. Calcium

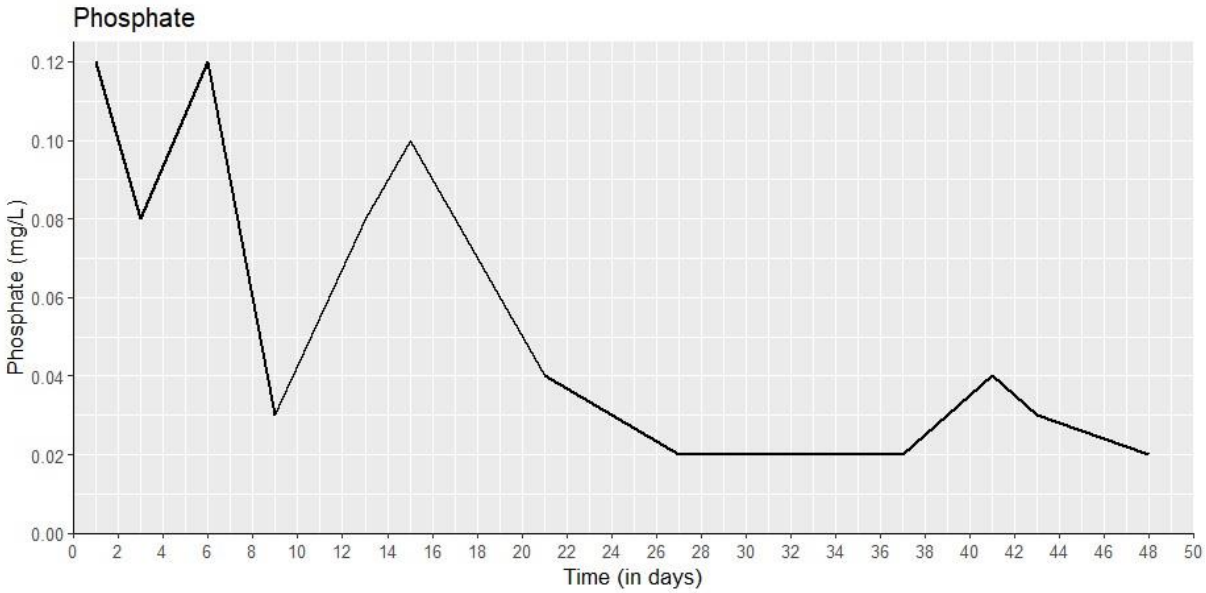



Fig. 18. Phosphate

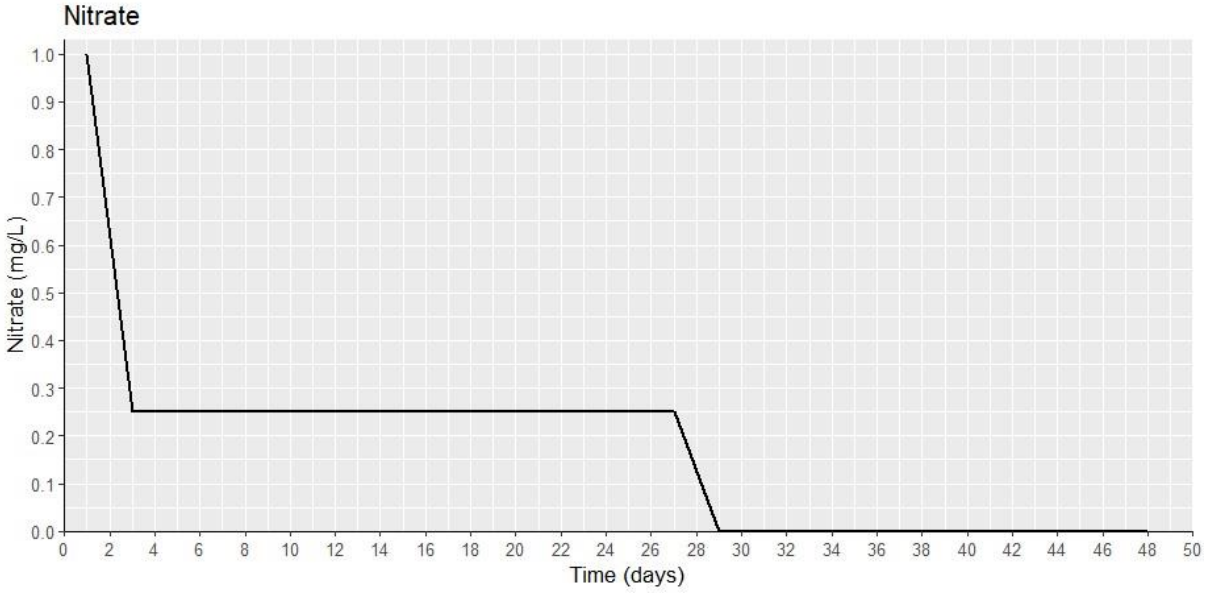



Fig. 19. Nitrate

## *Appendix 7: Logbook*

| Day | Date | Temp H | Temp C | Salinity H | Salinity C | Notes |
|---|---|---|---|---|---|---|
| 1 | 17-Apr | 26.1 | 26.2 | 35.3 | 35.3 | Put the corals inside the tanks, polyps seem to be a bit retracted possibly due to stress. WQ H5 & C6 |
| 2 | 18-Apr | 26.0 | 26.2 | 35.3 | 35.3 | First PAM (both avg 0.620). Polyps are less retracted |
| 3 | 19-Apr | 26.0 | 26.1 | 35.5 | 35.4 | WQ H1 & C8 |
| 4 | 20-Apr | | | | | |
| 5 | 21-Apr | | | | | |
| 6 | 22-Apr | 26.0 | 26.1 | 35.9 | 35.9 | Second PAM, WQ H7 & C2 |
| 7 | 23-Apr | 26.0 | 26.2 | 35.9 | 35.9 | Tanks cleaned and moved one spot towards the heater. Waterpik method successfully tested |
| 8 | 24-Apr | | | | | |
| 9 | 25-Apr | 25.9 | 26.1 | 35.9 | 35.8 | PAM. WQ H4 & C5 |
| 10 | 26-Apr | 26.2 | 26.4 | 36.0 | 35.9 | Start holiday |
| 11 | 27-Apr | | | | | |
| 12 | 28-Apr | | | | | |
| 13 | 29-Apr | 26.1 | 26.3 | 34.2 | 34.3 | PAM measured by Diederik |
| 14 | 30-Apr | 26.3 | 26.4 | 34.6 | 34.6 | |
| 15 | 01-May | 26.2 | 26.4 | 34.8 | 34.8 | Temperature increased to 27 |
| 16 | 02-May | 27.3 | 26.4 | 35.0 | 35.0 | PAM measured by Diederik. C1 fluorescent yellow. Power outage caused the pump to shut down for 3 hours, it wasn't mentioned. Fixed it when I didn't see water coming out the tubes |
| 17 | 03-May | 27.2 | 26.4 | 35.2 | 35.0 | |
| 18 | 04-May | | | | | |
| 19 | 05-May | | | | | |
| 20 | 06-May | 27.1 | 26.2 | 35.1 | 35.1 | End holiday. Temperature increased to 28. PAM |
| 21 | 07-May | 28.0 | 26.2 | 35.2 | 35.2 | Photo of C1 (fluorescent yellow). WQ H2 & C4 |
| 22 | 08-May | 28.0 | 26.3 | 35.3 | 35.2 | PAM, C1 top unmeasurable. Tanks cleaned and moved |
| 23 | 09-May | 28.1 | 26.3 | 35.4 | 35.4 | |
| 24 | 10-May | 28.0 | 26.3 | 35.5 | 35.5 | Temperature increased to 29. PAM, C1 top still unmeasurable. WQ H3 & C6 |
| 25 | 11-May | | | | | |
| 26 | 12-May | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 27 | 13-May | 29.0 | 26.4 | 35.7 | 35.7 | PAM, C1 top unmeasurable. WQ H8 & C1 |
| 28 | 14-May | 28.8 | 26.2 | 35.6 | 35.6 | Temperature increased to 30. Photos of tentacles. PAM C1 unmeasurable in dark |
| 29 | 15-May | 29.7 | 26.2 | 35.6 | 35.5 | PAM. WQ H2 & C7 |
| 30 | 16-May | 30.2 | 26.5 | 35.6 | 35.5 | Symbiont density |
| 31 | 17-May | 29.8 | 26.2 | 35.5 | 35.5 | PAM. WQ H6 & C3. Tanks moved |
| 32 | 18-May | | | | | Temperature increased to 31 |
| 33 | 19-May | | | | | |
| 34 | 20-May | 31.0 | 26.3 | 35.6 | 35.6 | |
| 35 | 21-May | 30.9 | 26.3 | 35.7 | 35.6 | From today daily PAM, top C1 measurable WQ H4 & C5 |
| 36 | 22-May | 31.1 | 26.4 | 35.6 | 35.6 | |
| 37 | 23-May | 31.0 | 26.3 | 35.7 | 35.6 | WQ H5 & C4. Tanks cleaned and moved |
| 38 | 24-May | 30.9 | 26.3 | 35.6 | 35.4 | PAM, C1 top and bottom unmeasurable |
| 39 | 25-May | 30.9 | 26.3 | 35.5 | 35.4 | Bleaching started, visually heatwave corals seem more fluorescent yellow. Photos taken from H6 & H8 |
| 40 | 26-May | 30.9 | 26.3 | 35.5 | 35.4 | PAM declining |
| 41 | 27-May | 29.9 | 26.7 | 35.5 | 35.4 | In morning temp heatwave 1 degree lower and control 0.5 degree higher, around 13:00 temp is at 31. WQ H6 & C3 |
| 42 | 28-May | 30.1 | 26.4 | 35.5 | 35.4 | Corals fluorescent yellow/white. PAM at 0.4, multiple tops unmeasurable. Multiple power outages so temp was lower. Symbiont density (156000). Water samples collected for filtration |
| 43 | 29-May | 30.9 | 26.3 | 35.5 | 35.4 | PAM declined to 0.271. Photos taken. Water samples collected. WQ H2 & C7 |
| 44 | 30-May | 30.9 | 26.3 | 35.5 | 35.4 | PAM declined to 0.128. Symbiont density (33000). Water samples collected. Temp to 30 in the afternoon |
| 45 | 31-May | 30.0 | 26.3 | 35.4 | 35.4 | Temp to 29 |
| 46 | 01-Jun | 29.1 | 26.3 | 35.5 | 35.5 | Temp to 28 |
| 47 | 02-Jun | 28.2 | 26.4 | 35.6 | 35.5 | Temp to 27 |
| 48 | 03-Jun | 27.2 | 26.3 | 35.6 | 35.6 | Temp to 26. WQ H1 & C8 |
| 49 | 04-Jun | 26.2 | 26.2 | 35.6 | 35.5 | Half of corals placed back in main tank |
| 50 | 05-Jun | 26.3 | 26.3 | 35.6 | 35.6 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 51 | 06-Jun | 26.1 | 26.1 | 35.6 | 35.6 | Recovery PAM. WQ H3 & C6 |
| 52 | 07-Jun | 26.3 | 26.3 | 35.7 | 35.6 | |
| 53 | 08-Jun | | | | | |
| 54 | 09-Jun | | | | | |
| 55 | 10-Jun | 26.3 | 26.4 | 35.7 | 35.6 | |
| 56 | 11-Jun | | | | | |
| 57 | 12-Jun | 26.2 | 26.2 | 35.5 | 35.5 | Recovery PAM. WQ H5 & C4 |
| 58 | 13-Jun | | | | | |
| 59 | 14-Jun | 26.2 | 26.2 | 35.5 | 35.6 | |
| 60 | 15-Jun | | | | | |
| 61 | 16-Jun | | | | | |
| 62 | 17-Jun | 26.1 | 26.1 | 35.3 | 35.4 | WQ H4 & C5 |
| 63 | 18-Jun | | | | | |
| 64 | 19-Jun | 26.1 | 26.3 | 35.2 | 35.3 | Recovery PAM |
| 65 | 20-Jun | 26.2 | 26.3 | 35.2 | 35.3 | |
| 66 | 21-Jun | 26.1 | 26.3 | 35.3 | 35.4 | WQ H3 & C6 |
| 67 | 22-Jun | | | | | |
| 68 | 23-Jun | | | | | |
| 69 | 24-Jun | 26.2 | 26.2 | 35.4 | 35.4 | 2 control (C7 & C8) and 2 heatwave (H1 & H5) corals used for symbiont identification. Symbiont density |
| 70 | 25-Jun | | | | | |
| 71 | 26-Jun | 26.3 | 26.3 | 35.5 | 35.5 | Recovery PAM |

# *Appendix 8: R scripts*

## *8.1. Effective yield*

```
### Load libraries
library(readxl)
library(ggplot2)
library(car)
library(ggpubr)
library(tidyverse)
library(tidyr)


### Load the data
pam <- read_excel("pam data.xlsx") %>%

## transform into standard dataframe
pivot_longer(cols = c(Heatwave, Control), names_to = "Treatment", values_to = "Value")


# Plotting with ggplot2
ggplot(pam, aes(x = Day, y = Value, color = Treatment, shape = Treatment)) +
   geom_point(size = 2.4) +
   geom_line(aes(group = Treatment), size = 0.6, show.legend = FALSE) +
   labs(x = "Time (days)", y = "Mean PAM") +
   ggtitle("PAM") +
   scale_y_continuous(breaks = seq(0, 0.7, by = 0.1), limits = c(0, 0.7)) +
   scale_x_continuous(breaks = seq(0, 46, by = 2), limits = c(0, 46)) +
   coord_cartesian(expand = FALSE) +
   theme(axis.line = element_line())


day45 <- read_xlsx("Day 45.xlsx")

### Convert the cattle data into "long" format
day45 <- pivot_longer(day45,
                      cols = names(day45),
                      names_to = "treatment",
                      values_to = "pam",
                      names_transform = list(treament = as.factor))

day45

leveneTest(pam ~ treatment, data = day45, center = mean)

t.test(pam ~ treatment, data = day45, var.equal = FALSE)
```

*8.2. Symbiont density*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(stringr)  # To retrieve characters for the day column creation


### Load the data
symbionts <- read_excel("symbiont density.xlsx")

# explore
symbionts
glimpse(symbionts)
summary(symbionts)

symbionts$Time <- as.factor(symbionts $Time)
symbionts $Treatment <- as.factor(symbionts $Treatment)

### Test for normality
symbionts %>%
  group_by(treatment) %>%
  summarise(shapiroTest = shapiro.test(symbionts)$p.value)


### Test for equality of variances
leveneTest(symbionts ~ treatment, data = symbionts)

### Anova
aov(Symbionts ~ Time * Treatment, data = symbionts)
Test <- aov(Symbionts ~ Time * Treatment, data = symbionts)
summary(Test)

### Tukey posthoc test
HSD.test(lm_symbionts, trt = "treatment", group = FALSE, console = TRUE)

# Perform the HSD test and store the result in a df

hsd_result <- HSD.test(lm_symbionts, trt = "treatment",
                       group = TRUE, console = TRUE)$groups %>%
  rownames_to_column( var = "treatment")

library(dplyr)
library(ggplot2)

## Add columns for ggplot
groupped_symbionts <- symbionts %>%
mutate(time = str_extract(treatment, "\\w+ \\w+$"),
       type = word(treatment, 1)) %>%
  left_join(hsd_result%>%select(c(treatment,groups)))
```

```
# Summarise data with standard errors
symbionts_summary <- groupped_symbionts %>%
  group_by(treatment, type, time) %>%
  summarise(
    mean = mean(symbionts),
    sd = sd(symbionts),
    n = n(),
    se = sd / sqrt(n)
  ) %>%
  left_join(hsd_result%>%select(c(treatment,groups)))

ggplot(symbionts_summary, aes(x = time, y = mean, group = type, color = type)) +
  geom_point(aes(shape = type), size = 3) +  # Points with different shapes and black outline
  geom_line(size = 0.8) +  # Line connecting means, grey color
  geom_errorbar(aes(ymin = mean - se, ymax = mean + se), width = 0.2, size = 0.8) +  #
Error bars for standard error (se), black color
  geom_label(aes(label = groups), vjust = -0.9, color = "black", label.size = 0) +  # Labels for
groups, black color
  xlab("Time") +  # X-axis label
  ylab(expression("Symbiont density (cells cm"~""~""^"-2"~")")) +  # Y-axis label
  ggtitle("Symbiont Density") +  # Plot title
  scale_color_discrete(name = "Treatment") +  # Legend title for color (type), changed from
'type' to 'Treatment'
  scale_shape_discrete(name = "Treatment") +  # Legend title for shape (type), changed from
'type' to 'Treatment'
  scale_y_continuous(breaks = seq(0, 700000, by = 100000), limits = c(0, 700000), labels =
scales::comma,
                     expand = expansion(mult = c(0, 0.05))) +  # Y-axis scale with specific breaks
and limits, formatted with commas
  coord_cartesian() +  # Keep default expansion for x-axis
  theme(axis.line = element_line())  # Theme adjustment for axis lines
```

*8.3. GFP*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(stringr)

# Load the data
gfp <- read_excel("GFP.xlsx")

my_data$time <- as.factor(my_data$time)
my_data$treatment <- as.factor(my_data$treatment)

# Test for normality
normality_tests <- gfp %>%
  group_by(treatment) %>%
  summarise(shapiroTest = shapiro.test(gfp)$p.value)

print(normality_tests)

# Test for equality of variances
levene_test <- leveneTest(gfp ~ treatment, data = gfp)
print(levene_test)

# ANOVA
aov(GFP ~ Time * Treatment, data = gfp)

Test <- aov(GFP ~ Time * Treatment, data = gfp)
summary(Test)

# Tukey posthoc test
tukey_result <- HSD.test(Test, trt = "treatment", group = FALSE, console = TRUE)
print(tukey_result)

# Perform the HSD test and store the result in a df
hsd_result <- HSD.test(Test, trt = "treatment",
                       group = TRUE, console = TRUE)$groups %>%
  rownames_to_column(var = "treatment")

library(dplyr)
library(ggplot2)

# Assuming gfp and hsd_result are properly defined
# Add columns for ggplot
groupped_gfp <- gfp %>%
  mutate(time = str_extract(as.character(treatment), "\\w+ \\w+$"),
         type = word(as.character(treatment), 1))

str(hsd_result)
```

```
hsd_result_selected <- hsd_result %>%
  select("treatment", "groups")  # or select(treatment, groups) without quotes

# Left join with gfp
gfp_with_groups <- gfp %>%
  left_join(hsd_result_selected, by = "treatment")

# Print or inspect the modified gfp_with_groups data frame
print(gfp_with_groups)

# Left join with groupped_gfp
groupped_gfp <- left_join(groupped_gfp, hsd_result_selected, by = "treatment")

# Print or inspect the modified data frame
print(groupped_gfp)

# Summarise data with standard errors
gfp_summary <- groupped_gfp %>%
  group_by(treatment, type, time) %>%
  summarise(
    mean = mean(gfp),
    sd = sd(gfp),
    n = n(),
    se = sd / sqrt(n)
  ) %>%
  left_join(hsd_result%>%select(c(treatment,groups)))

ggplot(gfp_summary, aes(x = time, y = mean, group = type, color = type)) +
  geom_point(aes(shape = type), size = 3) +  # Points with different shapes and black outline
  geom_line(size = 0.8, aes()) +  # Line connecting means, grey color
  geom_errorbar(aes(ymin = mean - se, ymax = mean + se), width = 0.2, size = 0.8) +  #
Error bars for standard error (se), black color
  geom_label(aes(label = groups), vjust = -0.9, color = "black", label.size = 0) +  # Labels for
groups, black color
  xlab("Time") +  # X-axis label
  ylab(expression("GFP")) +  # Y-axis label
  ggtitle("GFP") +  # Plot title
  scale_color_discrete(name = "Treatment") + # Legend title for color (type), changed from
'type' to 'Treatment'
  scale_shape_discrete(name = "Treatment") +
  scale_y_continuous(breaks = seq(0, 20000, by = 5000), limits = c(0, 20000), labels =
scales::comma) +
  coord_cartesian() +  # Keep default expansion for x-axis
  theme(axis.line = element_line())  # Theme adjustment for axis lines
```

*8.4. Temperature*

```
### Load libraries
library(readxl)
library(tidyverse)
library(ggplot2)


### Load the data
temp <- read_excel("temperature.xlsx") %>%

  ## transform into standard dataframe
  pivot_longer(cols = c(Heatwave, Control), names_to = "Treatment", values_to = "Value")

y_breaks <- seq(26, 31.5, by = 0.2)[-c(1, 2)]
y_labels <- as.character(y_breaks)


# Plotting with ggplot2
plt <- ggplot(temp, aes(x = Day, y = Value, color = Treatment, shape = Treatment)) +
  geom_point(size = 2.4) +
  geom_line(aes(group = Treatment), size = 0.6, show.legend = FALSE) +
  labs(x = "Time (days)", y = "Temperature (°C)") +
  ggtitle("Temperature") +
  scale_y_continuous(breaks = seq(25, 31.5, by = 0.5), limits = c(25, 31.5)) +
  scale_x_continuous(breaks = seq(0, 51, by = 2), limits = c(0, 51)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())

gt <- ggplotGrob(plt)

is_yaxis <- which(gt$layout$name == "axis-l")
yaxis <- gt$grobs[[is_yaxis]]

# You should grab the polyline child
yline <- yaxis$children[[1]]

yline$x <- unit(rep(1, 4), "npc")
yline$y <- unit(c(0, 0.06, 1, 0.1), "npc")
yline$id <- c(1, 1, 2, 2)
yline$arrow <- arrow(angle = 90)

yaxis$children[[1]] <- yline

gt$grobs[[is_yaxis]] <- yaxis

# grid plotting syntax
grid.newpage(); grid.draw(gt)
```

*8.5. Salinity*

```
### Load libraries
library(readxl)
library(tidyverse)
library(tidyr)
library(ggplot2)
library(scales)
library(grid)


### Load the data
sal <- read_excel("salinity.xlsx") %>%

  ## transform into standard dataframe
  pivot_longer(cols = c(Heatwave, Control), names_to = "Treatment", values_to = "Value")

y_breaks <- seq(34, 36.2, by = 0.2)[-c(1, 2)]
y_labels <- as.character(y_breaks)


# Plotting with ggplot2
plt <- ggplot(sal, aes(x = Day, y = Value, color = Treatment, shape = Treatment)) +
  geom_point(size = 2.4) +
  geom_line(aes(group = Treatment), size = 0.6, show.legend = FALSE) +
  labs(x = "Time (days)", y = "Salinity (ppt)") +
  ggtitle("Salinity") +
  scale_y_continuous(breaks = seq(33.6, 36.2, by = 0.2), limits = c(33.6, 36.2)) +
  scale_x_continuous(breaks = seq(0, 52, by = 2), limits = c(0, 52)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())

gt <- ggplotGrob(plt)

is_yaxis <- which(gt$layout$name == "axis-l")
yaxis <- gt$grobs[[is_yaxis]]

# You should grab the polyline child
yline <- yaxis$children[[1]]

yline$x <- unit(rep(1, 4), "npc")
yline$y <- unit(c(0, 0.06, 1, 0.1), "npc")
yline$id <- c(1, 1, 2, 2)
yline$arrow <- arrow(angle = 90)

yaxis$children[[1]] <- yline

gt$grobs[[is_yaxis]] <- yaxis

# grid plotting syntax
grid.newpage(); grid.draw(gt)
```

*8.6. Alkalinity*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(tidyr)
library(ggplot2)
library(scales)
library(grid)


### Load the data
alkalinity <- read_excel("alkalinity.xlsx")


y_breaks <- seq(3, 4, by = 0.1)[-c(1, 2)]
y_labels <- as.character(y_breaks)


# Plotting with ggplot2
plt <- ggplot(alkalinity, aes(x = Day, y = Alk)) +
  geom_line(size = 0.8) +
  labs(x = "Time (days)", y = "Alkalinity (mEq/L)", title = "Alkalinity") +
  ggtitle("Alkalinity") +
  scale_y_continuous(breaks = seq(2.8, 4, by = 0.1), limits = c(2.8, 4)) +
  scale_x_continuous(breaks = seq(0, 50, by = 2), limits = c(0, 50)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())

gt <- ggplotGrob(plt)

is_yaxis <- which(gt$layout$name == "axis-l")
yaxis <- gt$grobs[[is_yaxis]]

# You should grab the polyline child
yline <- yaxis$children[[1]]

yline$x <- unit(rep(1, 4), "npc")
yline$y <- unit(c(0, 0.06, 1, 0.1), "npc")
yline$id <- c(1, 1, 2, 2)
yline$arrow <- arrow(angle = 90)

yaxis$children[[1]] <- yline

gt$grobs[[is_yaxis]] <- yaxis

# grid plotting syntax
grid.newpage(); grid.draw(gt)
```

*8.7. Calcium*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(tidyr)
library(ggplot2)
library(scales)
library(grid)


### Load the data
calcium <- read_excel("calcium.xlsx")

y_breaks <- seq(350, 450, by = 0.2)[-c(1, 2)]
y_labels <- as.character(y_breaks)


# Plotting with ggplot2
plt <- ggplot(calcium, aes(x = Day, y = Ca)) +
  geom_line(size = 0.8) +
  labs(x = "Time (days)", y = "Calcium (mg/L)", title = "Calcium") +
  ggtitle("Calcium") +
  scale_y_continuous(breaks = seq(330, 450, by = 10), limits = c(330, 450)) +
  scale_x_continuous(breaks = seq(0, 50, by = 2), limits = c(0, 50)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())

gt <- ggplotGrob(plt)

is_yaxis <- which(gt$layout$name == "axis-l")
yaxis <- gt$grobs[[is_yaxis]]

# You should grab the polyline child
yline <- yaxis$children[[1]]

yline$x <- unit(rep(1, 4), "npc")
yline$y <- unit(c(0, 0.06, 1, 0.1), "npc")
yline$id <- c(1, 1, 2, 2)
yline$arrow <- arrow(angle = 90)

yaxis$children[[1]] <- yline

gt$grobs[[is_yaxis]] <- yaxis

# grid plotting syntax
grid.newpage(); grid.draw(gt)
```

### *8.8. Phosphate*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(tidyr)

### Load the data
phosphate <- read_excel("phosphate.xlsx")

library(ggplot2)

# Plotting with ggplot2
ggplot(phosphate, aes(x = Day, y = PO4)) +
  geom_line(size = 0.8) +
  labs(x = "Time (in days)", y = "Phosphate (mg/L)", title = "Phosphate") +
  scale_y_continuous(breaks = seq(0, 0.125, by = 0.02), limits = c(0, 0.125)) +
  scale_x_continuous(breaks = seq(0, 50, by = 2), limits = c(0, 50)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())
```

### *8.9. Nitrate*

```
### Load libraries
library(FSA)
library(agricolae)
library(fitdistrplus)
library(ggh4x)
library(car)
library(readxl)
library(tidyverse)
library(tidyr)

### Load the data
nitrate <- read_excel("nitrate.xlsx")

library(ggplot2)

# Plotting with ggplot2
ggplot(nitrate, aes(x = Day, y = NO3)) +
  geom_line(size = 0.8) +
  labs(x = "Time (days)", y = "Nitrate (mg/L)", title = "Nitrate") +
  scale_y_continuous(breaks = seq(0, 1.03, by = 0.1), limits = c(0, 1.03)) +
  scale_x_continuous(breaks = seq(0, 50, by = 2), limits = c(0, 50)) +
  coord_cartesian(expand = FALSE) +
  theme(axis.line = element_line())
```