Effects of elevated temperatures and health status of the crustose coralline algae *Hydrolithon boergesenii* (Corallinales: Mastophoroideae) on the larval settlement of *Diploria labyrinthiformis* (Scleractinia: Faviinae)

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Abstract

Crustose Coralline Algae (CCA) is a well-known settlement inducer for stony corals and, ultimately, recruitment, a vital component for reef growth and resilience. However, potential impacts of diseased CCA on larval settlement are not fully understood, especially on particular coral species. As oceans continue to warm, coral larvae need to be able to respond to settlement cues in elevated temperatures, yet the combined effects of thermal stress and CCA health status on larval behavior is not well known for most coral species. Here we assessed the effect of elevated temperatures and disease on the ability of the CCA *Hydrolithon boergesenii* to induce settlement of *Diploria labyrinthiformis* larvae. *D. labyrinthiformis* planulae were exposed to 4 substrate combinations (healthy CCA, diseased CCA, bare substratum, and bare tissue culture plate) and three temperatures (27.5°C, 29°C, and 31°C). Overall, settlement started earlier and was 1.5-3x higher at 31°C, regardless of CCA health status, but at this temperature, larval mortality increased two-fold in diseased CCA. Settlement differences between healthy and diseased *H. boergesenii* were only observed at 29°C, with healthy CCA facilitating twice as much settlement and having 3x lower mortality than diseased. Our findings suggest that, even though larvae can settle in both healthy and diseased CCA, temperature plays an important role in whether larvae will settle or perish. This study highlights the importance of healthy CCA to maintain and increase settlement and the ability of larvae to adapt to a warming ocean, contributing to the knowledge of *D. labyrinthiformis* larval ecology, valuable for larval rearing for restoration purposes.

Introduction

Caribbean coral populations have been declining for decades due to a combination of local and global stressors such as increasing sea surface temperatures, nutrient pollution, diseases, and coastal development (Hughes 1994; Hughes et al. 2010; de Bakker et al. 2017). Consequently, coral restoration efforts have become more widespread in recent years, acknowledging that coral reefs are unlikely to recover without human intervention (Morrison et al. 2019; Muñiz-Castillo et al. 2019). However, these efforts still produce outcomes with varying degrees of success, limited by high costs and a relatively low impact on an ecosystem level and at the spatial scales needed (Bayraktarov et al. 2016, 2019; Boström-Einarsson et al. 2020; Duarte et al. 2020).

Coral recruitment can be used as a proxy for reef resilience and recovery since it yields novel genotypes that can potentially provide greater adaptability to unfavorable conditions (Baums et al. 2019). However, for most coral species, little is known about corals' early life stages i.e., the period when these organisms have higher mortality rates (Vermeij and Sandin 2008; Doropoulos et al. 2016).This is especially true for the pre-settlement period, when larvae swim in the water column seeking an appropriate substrate to settle, since they can perish due to energy storage depletion (Vermeij 2006). Studying the early life stage ecology of specific coral species is key for understanding its contribution to the current and future composition of reefs (Chamberland et al. 2016). Therefore, identifying the factors that affect larval recruitment success can inform management decisions aimed at reducing the decline of coral reefs and improve restoration efforts through sexual propagation.

Crustose Coralline Algae (CCA) is one of the main contributors to reef cementation and has been shown to induce coral larval settlement (Pollock et al. 2017). However, according to Quéré et al. 2015, the emergence of CCA diseases such as Coralline White Band Syndrome (CWBS) and Coralline White Patch Disease (CWPD), both significantly affected by temperature changes, can pose a real threat to corals by reducing the settlement of their offspring. Thus, the rapid emergence of diseases affecting reef organisms and the more frequent and severe bleaching events in an era of rapid ocean warming may jeopardize the natural capacity of coral reefs to recover from disturbances (Hoegh-Guldberg et al. 2007; Baker et al. 2008).

In this study, we assessed the effects of CCA health condition and high temperatures on larval settlement of the coral *Diploria labyrinthiformis* (Linnaeus, 1758). For this, we conducted a short-term controlled experiment on *D. labyrinthiformis* larvae exposed to different temperatures (expected summer temperatures (27–28°C) and two increased temperature scenarios (29°C and 31°C)) and using healthy and diseased *Hydrolithon boergesenii* (Guiry and Guiry 2022) for settlement induction. We expected higher settlement rates in the presence of healthy CCA and in their optimum rearing temperature of ~ 27.5°C.

Materials And Methods

Study species and site

Diploria labyrinthiformis is a common coral species in the Caribbean and, depending on its location, can spawn from April to October before sunset, having a wider and earlier spawning window compared to most broadcast spawning corals in the region (Weil and Vargas 2010; Chamberland et al. 2017). *D. labyrinthiformis* was selected for this study due to its ability to build 3D structures in reefs, its early and multiple spawning events throughout the year, its high spawning predictability, and the contribution to recent and ongoing research regarding its reproductive potential and early life history stages (Chamberland et al. 2016). FUNDEMAR has been monitoring and documenting spawning events of this species at the Playita reef site since May 2017, producing a spawning prediction calendar for 2020 including this and 7 other coral species (Sellares-Blasco et al. 2021). Currently, there are over 150 *D. labyrinthiformis* adult colonies identified and tagged on this site to monitor individual colony spawning activity through time.

Hydrolithon boergesenii is a Crustose Coralline Algae (CCA) species of the subfamily Mastophoroideae that grows encrusted in the rubble and as a rhodolite in high energy shallow habitats ranging from 1–70 m depth. It has a light purple coloration that can vary depending on light intensity. It is composed of small, abundant, and elevated conceptacles with a dome shape, tessellar texture, scattered trichocytes, and a single layer hypothallus (Minnery 1990; Amado-Filho et al. 2018). Several scientists have demonstrated that *Hydrolithon boergesenii* induces larval settlement in spawning corals such as *D. labyrinthiformis* (Quéré et al. 2015), *Pseudodiploria strigosa* (Ritson-Williams et al. 2016), *Acropora*

cervicornis and *Acropora palmata* (Ritson-Williams et al. 2010); and brooding corals such as *Agaricia humilis* (Raimondi and Morse 2000), *Favia fragum* and *Porites astreoides* (Ritson-Williams et al. 2016).

The experiment was conducted in the Dominican Foundation for Marine Studies' (FUNDEMAR) Coral Assisted Reproduction Laboratory, located in Bayahibe, Dominican Republic (18°21’57.07” N 68°50’29.439” W) (Fig. 1). *Hydrolithon boergesenii* samples were taken from the El Sombrero site (18°22'14.1"N 68°50'48.3"W), a highly degraded shallow reef (also a restoration site for FUNDEMARs restoration program) located 500 m from the coast of Bayahibe, with an average depth of 4 m. CCA was found in high-energy areas where waves were breaking on the reef and *Hydrolithon boergesenii* is conspicuous. This makes it an ideal place to serve as a reference for the purpose of this study. *Diploria labyrinthiformis* gamete bundles were collected at the Playita site (18°22'23.1"N 68°51'11.7"W), a local reef flat located 750 m from El Sombrero site and 550 m off the coast of Bayahibe with an average depth of 6.4 m. This reef has a moderate relative abundance (4.23 ± 3.61%) and high density (0.33 ± 0.2/m^2) of adult *D. labyrinthiformis* colonies.

Larvae production and substratum preparation

Diploria labyrinthiformis colonies at Playita *s*pawned on June 16, 2020, 11 nights after the full moon and 75 to 50 minutes before sunset. From the 50 colonies monitored, 17 spawned (34%). Gamete bundles were collected from 11 colonies using funneled nets with plastic collection cups on top (Fig. S1A-B). The cups with gametes were transported to the lab and mixed together to begin fertilization. Cellular division was assessed under a dissecting microscope every 20 minutes until a fertilization rate of 96% was reached (Fig. S1C). At this stage, embryos were rinsed of excess sperm to reduce mortality.

Finally, embryos were transferred to 9 closed glass aquariums (76.2 cm x 30.48 cm x 35.56 cm L, W, H each) filled with filtered seawater and maintained at around 27–28ºC for cultivation (Sellares-Blasco et al. 2021). Embryo culture maintenance was carried out daily, depending on culture condition, including lipid removal using plastic wrap and 50% water changes. Embryos reached the planulae phase approximately 21 hours after fertilization (Fig. S1D). Once larvae started to swim down the water column, around 36 hours after fertilization, individual larvae were randomly selected from a mix of samples from the 9 aquariums to use in this experiment.

Hydrolithon boergesenii was collected from Sombrero, where waves break with the reef crest. An initial visual ID of the species was done by using a Talbot 90 mm 5x Magnifying Glass. Then, a Pit Bull CHIH058 Chipping Hammer was used to remove the algae from the rock and samples were placed in plastic bags. Finally, a multi-purpose mesh bag was used for transportation to the lab. The algae samples were kept in a 5-gallon Marina LED Aquarium with filtered seawater. Another visual ID was done to confirm algae species with Ritson-Williams’s CCA ID Guide (2018, unpublished). After being identified, algae samples were cut to ~ 1 cm^2 with bone cutters from the DR Instruments 10FK Ultimate Coral Fragging Kit to be used as replicate substrates for the experiment.

Experimental design and statistical analysis

We performed a factorial orthogonal experiment with three factors: (1) Substrate type (4 levels of treatments), (2) temperature (3 levels of treatment) and (3) time. The experiment consisted of 9 polystyrene culture plates with 12 wells (EarthOx Life Sciences TCPN12 Sterile Non-Treated 12 Well Tissue Culture Plate). Each plate was randomly assigned with 3 replicates of 4 substrate treatments: bare substratum, calcium carbonate skeleton with large macroalgae removed but some turf algae remaining, as control (CO), healthy CCA (CCAH), diseased CCA (CCAD), and empty culture wells as procedural control (PC). Culture plates were then independently and randomly exposed to 3 temperature treatments (27 ± 0.58°C, the optimum larval rearing temperature according to Banaszak (2018, unpublished), 29 ± 0.43°C, the average ocean temperature during the time of the experiment, and 31 ± 0.28°C, mimicking a substantial ocean warming scenario for the mid- 21st century), placing them in a water bath inside plastic polypropylene trays with filtered seawater. The trays were connected in a closed circuit of constant flow and a titanium heater with a digital thermostat controller of 100W to control the temperatures for each level. The water temperature of each system was manually measured daily every two hours from 7 am to 7 pm using a Thomas Traceable Kangaroo digital thermometer. The time frame of these measurements was limited by a curfew stated by the national government of the Dominican Republic due to Covid-19 restriction. A graphic of the experimental layout and experiment set up is provided in Fig. 2.

Around 36 hours after fertilization, once the larvae were competent to settle, a sample of planulae was randomly taken from each rearing aquarium, and they were all mixed in a container. A total of 10 to 12 larvae sampled randomly from this mix were assigned to each well of the 9 culture plates, resulting in 108 wells with 1,103 larvae. Data collection regarding the condition of the larvae (swimming, settled, floating, or dead) started 60 h after fertilization and 24 hours after the addition of larvae to the experimental set up. Condition was assessed once every morning from day 1 to day 7, except on day 2 since, when handling for assessment, larvae that were starting to settle were observed detaching from the substrate; therefore, we decided to leave them for one day without disruption.

We classified larvae as “settled” if any degree of metamorphosis was observed, starting with skeleton formation up to tentacle formation (Fig. 3A-F). Larvae that were still alive and motile but not attached, were classified as “swimming”; metamorphosed, unattached polyps, not unusual in lab culture settings, were classified as “floating” (Miller et al. 2020; Margaret Miller pers comms)(Fig. 3F); and larvae that were absent were classified as “dead”. Only settled and dead larvae were used to estimate settlement and mortality, respectively. Observations were made using a Nightsea FL-1 blue flashlight and yellow filter glasses to better locate fluorescing larvae. Settlement was confirmed under a dissecting microscope (Motic SMZ-168 Stereo Zoom microscope).

A univariate PERMANOVA based on Euclidean distance was performed to test for differences in larval settlement and mortality rates for each factor and all interactions. PERMANOVAs for settlement and mortality rates were run on untransformed percentage data using 9999 permutations under an unrestricted method and type III sum of square approach for unbalanced data. When a source of variation was significant ($P_{perm} < 0.05$), we performed a two-tailed pairwise t-test to detect statistically significant differences (Anderson 2001). Test of homogeneity of dispersion was performed using

Permdisp prior to running the Permanova test (Anderson and Walsh 2013). Dispersion of data was homogenous for each treatment.

Results And Discussion

In this study we found that *Diploria labyrinthiformis* larvae are affected by factors that interact to modify the outcome of settlement and/or mortality within a short period. Our results show that the interaction between time and temperature ($F = 2.13$, $df = 10$, $p = 0.012$), and between CCA condition and temperature ($F = 4.18$, $df = 6$, $p = 0.001$, Table 1A) significantly affected larval settlement. Combined, temperature, CCA condition and time explained over 63% of the total variance (Table 1A). On average, < 28% of the larvae settled within 7 days (the duration of this experiment), with significantly higher averages at day 6 and 7 at 31ºC (Fig. 4A, Table S1).

We found that *D. labyrinthiformis* copes well in temperatures above 27.5ºC, and such an increase does not seem to affect larval performance at least for a 7-day period. On the contrary, higher settlement rates were observed in the high temperature treatment (Fig. 4, Table S1). While increased temperatures could be detrimental for larvae of some coral species before and after settlement (Bassim and Sammarco 2003), it also may increase metabolic rates and promote faster development and early settlement (Nozawa and Harrison 2007; Munday et al. 2009; Randall and Szmant 2009; Heyward and Negri 2010). Other studies have also shown higher larval settlement in stressful conditions and interpreted it as an escape attempt that may affect survival in later life stages instead of an advantage (Vermeij et al. 2006). Furthermore, some have also described a carry-over or latent effect of temperature stress, where short-term larval survival, settlement, and metamorphosis were not affected by increased temperature, but, post-settlement, spats displayed a significant reduction in survival (Ross et al. 2013). However, in this study, post-settlement survival of *D. labyrinthiformis* spats could not be assessed since there were no surviving settlers after outplanting to an underwater nursery. Thus, future experiments with *D. labyrinthiformis* should focus on testing potential post-settlement effects of increased temperature exposure for this species.

On average, larval settlement was significantly higher for wells bearing CCA (healthy or diseased) compared to other substrates, particularly at 31ºC treatment. Settlement on healthy over diseased CCA was only significantly higher for the 29ºC treatment, further indicating the effect of CCA health status relies heavily on temperature (Fig. 4B, Table S1). Several species of CCA have been shown to promote settlement, while others deter it (Raimondi and Morse 2000; Ritson-Williams et al. 2009, 2016; Price 2010; Quéré et al. 2015). Diseases affecting CCA have been a concern for years because chemical cues might be compromised and/or the suitable space for settlement may become limited (Quéré and Nugues 2015; Quéré et al. 2015). Our results indicate that diseases affecting CCA hamper settlement at temperatures of 29^{o}C. However, thermal stress events may prompt larvae to settle regardless of CCA health status, provided these events do not prolong enough to compromise the survival of coral spats, which are often susceptible to ocean warming (Randall and Szmant 2009). Larvae rarely settled on bare substratum

(control) and more often did so on the polystyrene culture plates (procedural control), yet 27.5ºC was not significantly different from 31ºC (Fig. 4B, Table S1).

In terms of mortality rates, we found significant interactions between time (days) and substrate type, and between temperature and substrate type (Table 1B, Fig. 5, Table S2). Higher mortality rates were recorded at the end of the experiment, 7 days after larvae were competent for settlement, with about 75% dead larvae in the control treatment (Fig. 5A). Moreover, an average of < 15% of the larvae died on the healthy CCA and empty culture plates (PO) treatments (Fig. 5), which shows that procedural control did not increase mortality compared to this treatment. The percentage of mortality was always significantly higher for the diseased CCA treatment compared to healthy CCA, and almost two-fold higher when compared to the CCA healthy treatment, consistent for all temperature treatments (Fig. 5B).

As for the interaction between the health status of CCA and temperature, we consistently found higher mortality rates associated with the control treatment (58% for 27.5 ºC, 46% for 29ºC and 61% for 31ºC), followed by diseased CCA (32% for 27.5ºC, 30% for 29ºC and 15% for 31ºC), healthy CCA (15% for 27.5ºC, 14% for 29ºC and 13% for 31ºC) and the procedural control (< 15% for all temperature treatments) (Fig. 5B). Thus, results from this study showed that within the first 7 days after reaching competence to settle, average mortality is two-four-fold higher compared to the percentage of settlement, further suggesting this period is a bottleneck of survivorship for *D. labyrinthiformis*. Other studies conducted for Caribbean (Rylaarsdam 1983; Vermeij and Sandin 2008; Alvarado-Chacon et al. 2020) and Indo Pacific corals show similar results (Babcock 1985; Babcock and Mundy 1996; Babcock et al. 2003; Wilson and Harrison 2005). Thus, our results are consistent with the finding that high mortality rates during the initial stages of their life cycle are a critical part of life-history traits in corals (Trapon et al. 2013).

In conclusion, this study shows that larval settlement of *D. labyrinthiformis* is affected by the interaction between temperature, CCA health status, and time. Settlement was highest at the last days of the study, and particularly important to note, is the reduction of settlement and increased mortality recorded for unhealthy CCA substrates. Based on this, larvae rearing practices with *D. labyrinthiformis* can be benefitted by letting larvae settle for at least 7 days and by avoiding the use of diseased CCA in cultures. Although a temperature increase did foster larval settlement regardless of CCA health state, we would not advice to rear larvae over 28ºC since it could have potential detrimental latent or carry-over post-settlement effects that were not assessed in this study.

This study demonstrates the importance of healthy Crustose Coralline Algae communities to maintain and increase coral settlement in a warming ocean and adds to the knowledge of *D. labyrinthiformis* larval ecology under varying conditions, providing valuable information to optimize the culturing process for restoration purposes.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Data availability

The datasets generated during and/or analyzed during the current study are included in this published article in the supplementary information [Supplementary File 2- Data Matrix].

Ethics approval

Coral gametes were collected under permits issued by the Dominican Republic Ministry of Environment and Natural Resources issued on June 1st, 2020.

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Tables

Table 1. PERMANOVA results of A) larval settlement and B) larval mortality testing for differences between Substrate type (Su), Days (Da) and Temperature (Te) treatments and their interactions.

A. SETTLEMENT							
Source	df	SS	MS	Pseudo-F	P(perm)	perms	CV%
Substrate	3	8062.4	2687.5	34.554	0.001	998	16.26337
Days	5	10146	2029.2	26.091	0.001	999	17.22452
Temperature	2	4489	2244.5	28.859	0.001	998	12.8337
SuxDa	15	1374.6	91.639	1.1783	0.278	997	2.903589
SuxTe	**6**	**1954.4**	**325.74**	**4.1882**	**0.001**	**999**	**8.683172**
DaxTe	**10**	**1663.6**	**166.36**	**2.139**	**0.012**	**998**	**6.356476**
SuxDaxTe	30	1515.8	50.525	0.64964	0.923	998	0
Residual	576	44798	77.775				35.73517
Total	647	74004					
B. MORTALITY							
Substrate	3	2.16E+05	71902	169.67	0.001	999	28.01462
Days	5	1.05E+05	21093	49.773	0.001	999	18.45057
Temperature	2	3242.4	1621.2	3.8256	0.016	999	3.140225
SuxDa	**15**	**47745**	**3183**	**7.511**	**0.001**	**999**	**13.4825**
SuxTe	**6**	**18830**	**3138.4**	**7.4058**	**0.001**	**998**	**9.456286**
DaxTe	10	1654.7	165.47	0.39047	0.935	999	0
SuxDaxTe	30	7382.8	246.09	0.58071	0.965	997	0
Residual	576	2.44E+05	423.78				27.4558
Total	647	6.44E+05					

Figures

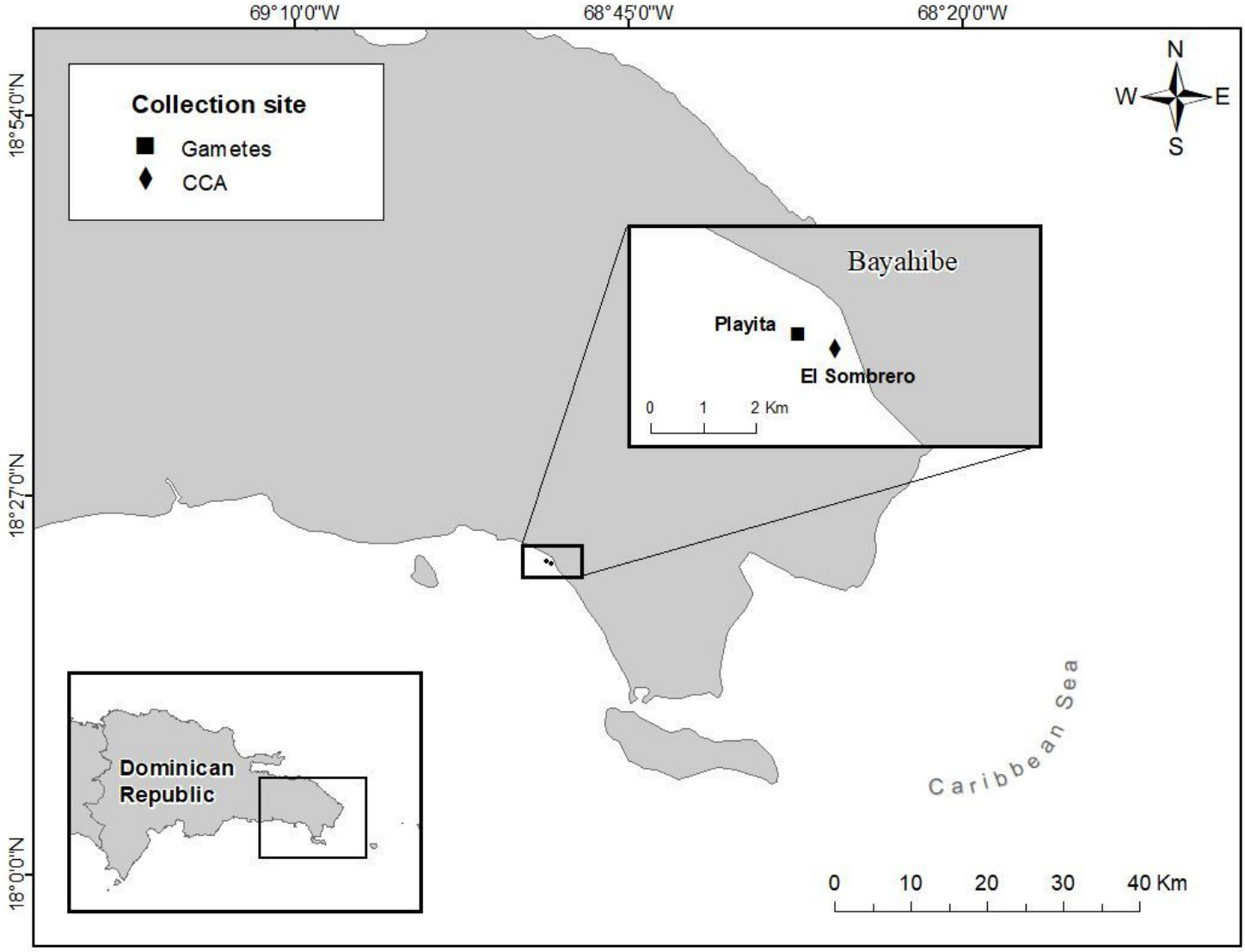

Figure 1

Map of southeastern Dominican Republic indicating the location of El Sombrero and Playita site where crustose coralline algae samples and gametes from *Diploria labyrinthiformis* were collected, respectively.

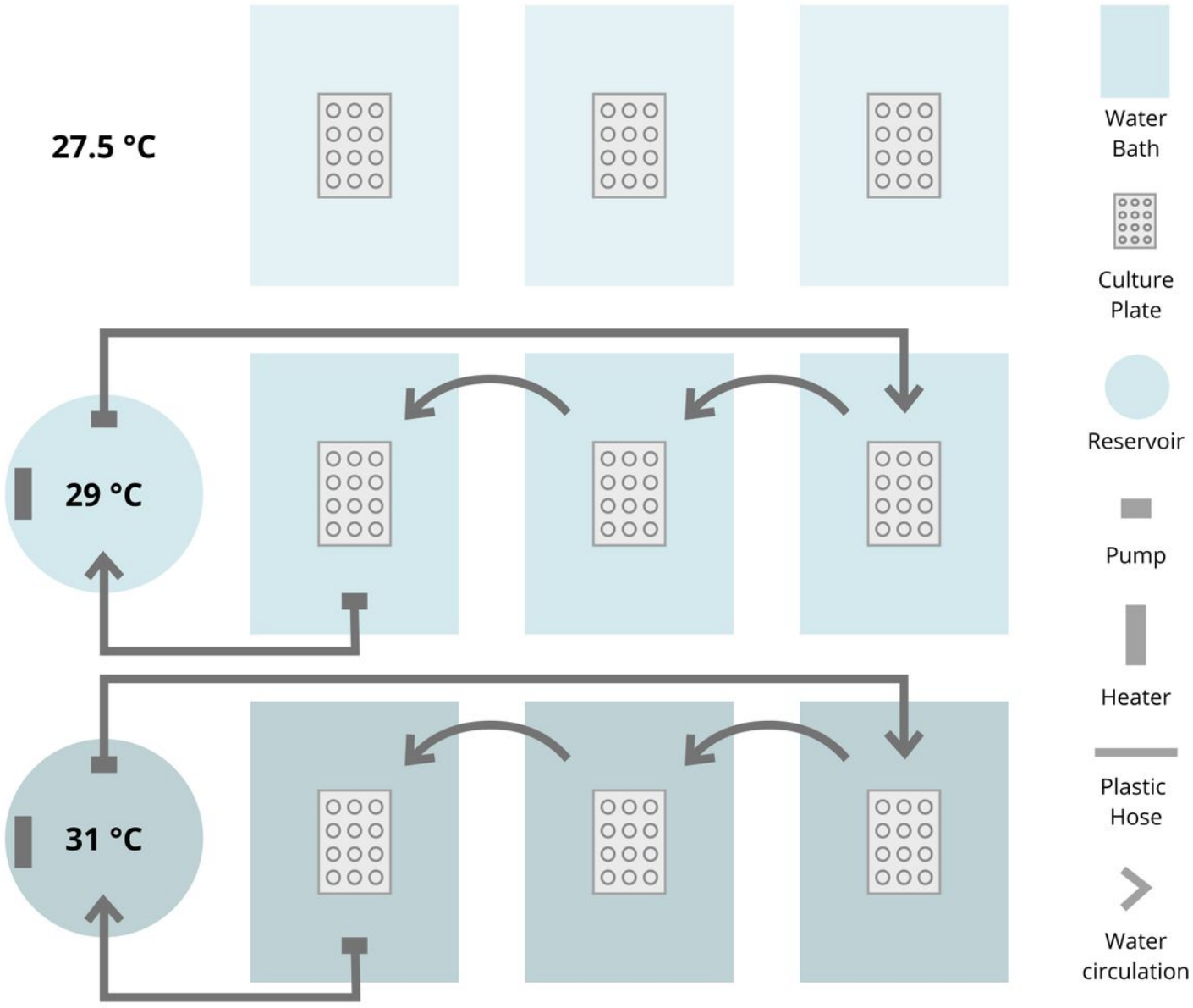

Figure 2

Experimental design and study set up. Substratum treatments (bare substratum as control (CO), healthy CCA (CCAH), diseased CCA (CCAD), and empty culture wells as procedural control (PC)) were replicated 3 times and randomly allocated within each polystyrene culture plate. Each plate was placed in an individual water tray with the different temperature treatment levels (ambient 27.5ºC, 29ºC, and 31ºC), each replicated 3 times. The trays with the same temperature treatment were connected to a reservoir where the water temperature was maintained by a heater.

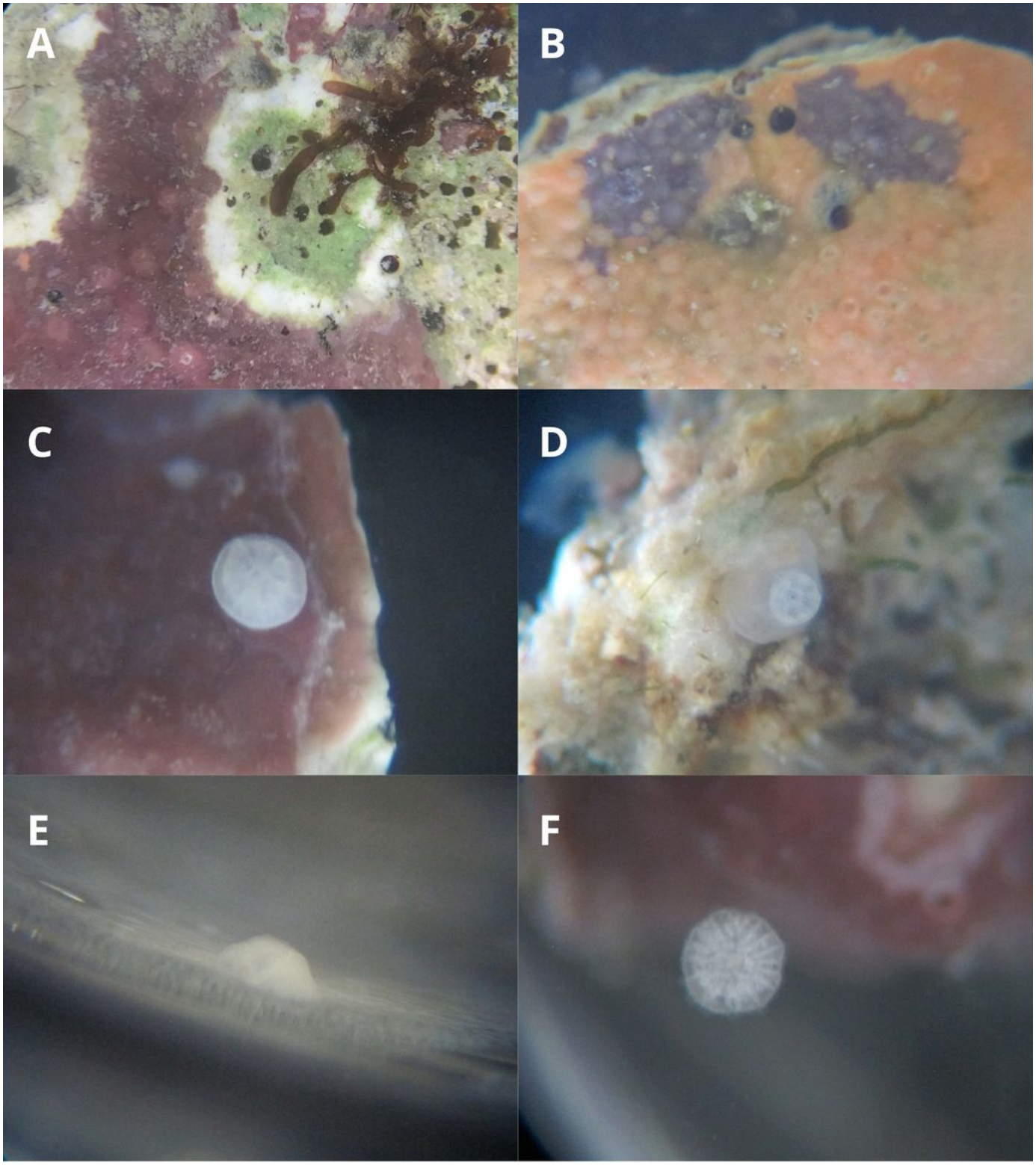

Figure 3

Substrate types provided to larvae: A and B) Diseased *Hydrolithon boergesenii* Crustose Coralline Algae (CCAD), C) Healthy CCA (CCAH), D) Bare substratum or calcium carbonate skeleton removed of large macroalgae with some turf algae remaining. Examples of larvae condition: C, D and E) settled and metamorphosed and F) metamorphosed, unattached polyps in the water column.

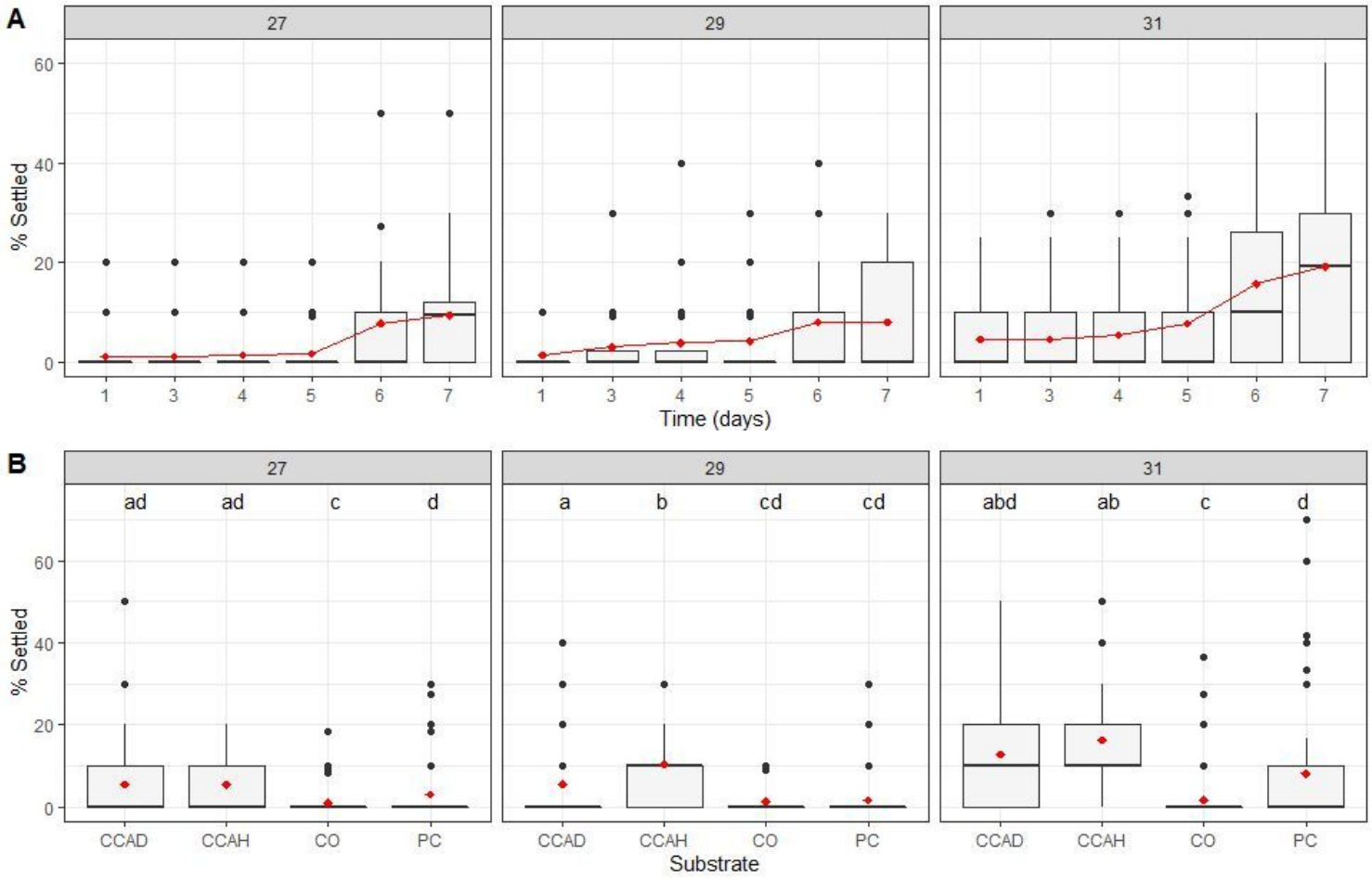

Figure 4

Settlement percentage of *D. labyrinthiformis* larvae in function of A) the seven days of the experiment and B) across experimental treatments (CCAD= Diseased crustose coralline algae, CCAH=Healthy crustose coralline algae, CO= Control, PC= Procedural control). Letters denote significant differences among experimental treatments from a post-hoc analysis. In both panels, red dots represent the mean values.

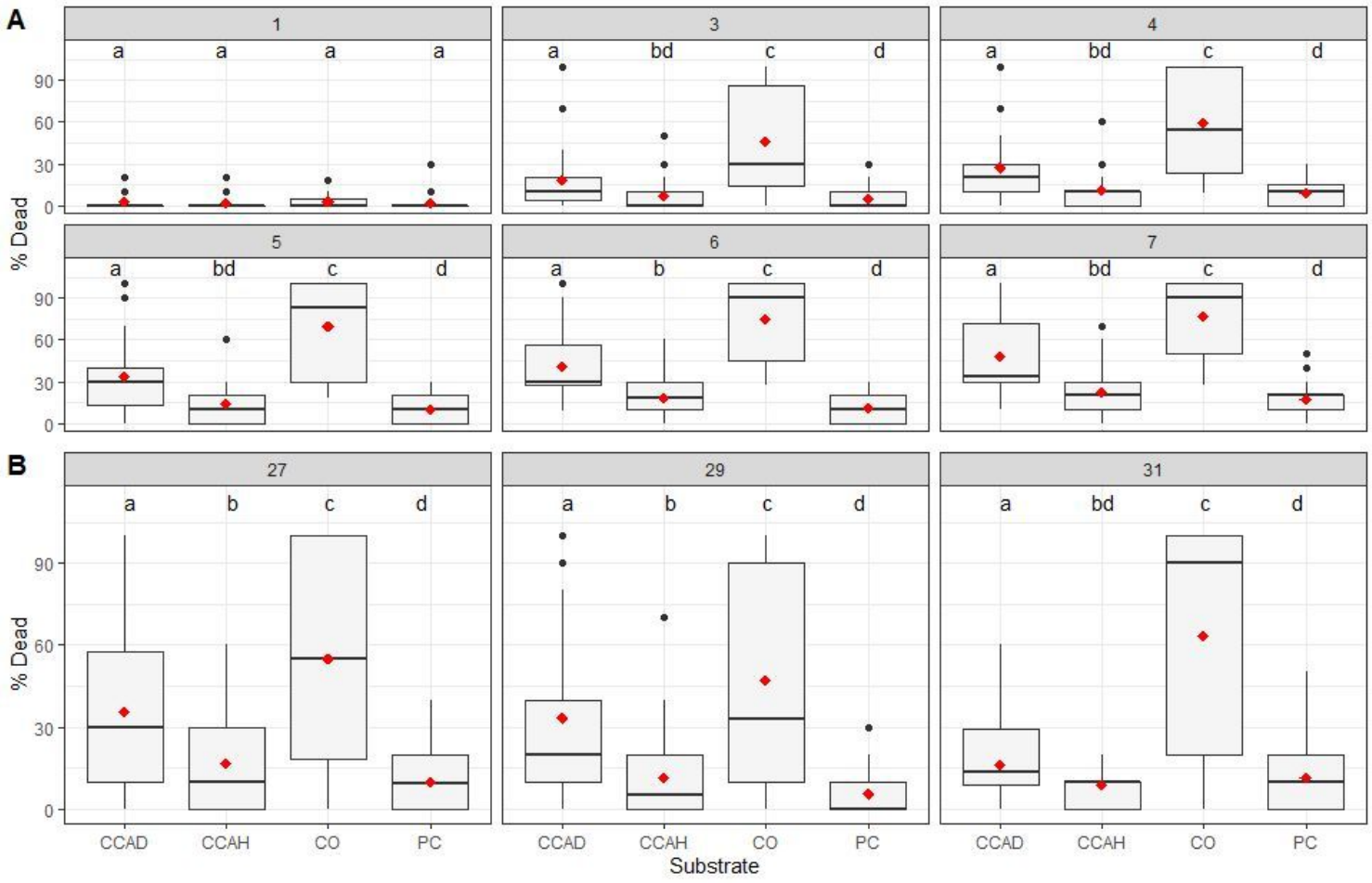

Figure 5

Percent mortality of *D. labyrinthiformis* larvae in function of A) treatments and the seven days of the experiment and B) across experimental treatments (CCAD= Diseased crustose coralline algae, CCAH=Healthy crustose coralline algae, CO= Control, PC= Procedural control). Letters denote significant differences among experimental treatments from a post-hoc analysis. In both panels, red dots represent the mean values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFile1.docx
- SupplementaryFile2DataMatrix.xlsx