The effect of *Sargassum* on settlement of *Diadema antillarum* larvae around Saba

Author: Jasper Bleijenberg (1030976)
Supervisors: Alwin Hylkema & Tinka Murk
Chair-group: Marine Animal Ecology
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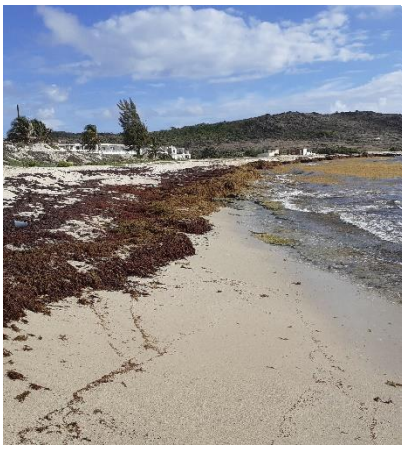
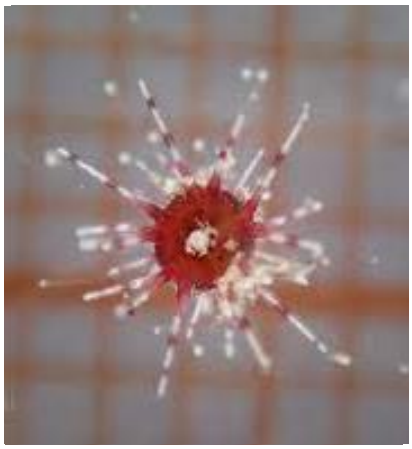

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Abstract

Before its mass-mortality in the 1980's, *Diadema antillarum* was the most important benthic herbivore on Caribbean reefs. Since then, reefs have experienced an increase in turf/macroalgae abundance. Despite the approximate 40 years that have past, natural recovery is slow and variable. Therefore efforts are taken to boost D. antillarum recovery artificially. Several bottlenecks for recovery have been found, but in the last decade a new potential problem has risen: massive influxes of floating *Sargassum* species. Studies have shown that algae or its biofilm can induce sea urchin settlement to a certain extent, but no in vivo experiments have been conducted for *D. antillarum*. Here we investigated if these *Sargassum* floats interfere with the recruitment of *D. antillarum* by measuring the abundance of *D. antillarum* settlers in submerged and floating, rinsed and unrinsed *Sargassum* units and comparing values with a positive control. While the amount of data collected with the low sample sizes did not suffice to answer the research question in any statistically significant manner, hypothesis could be formulated.

D. antillarum certainly is attracted to sargassum patches, whether as post-settler only or also as settler is however still debatable. Cues by conspecifics could possibly play a role in increased settlement in *Sargassum,* blurring the effect of *Sargassum* solely. A large variation in *D. antillarum* abundance was found for the unrinsed submerged treatment, which was possibly the result of migration from nearby existing ecosystems with older *D. antillarum* into the experimental units, or it was an artefact of the low sample size. To confidently distinguish signal from noise in a similar study, some alterations to the research design need to be made and drifting sargassum patches need to be sampled to get a sense of *D. antillarum* settler abundance on naturally occurring *Sargassum*.

Introduction

1.1 Problem description

The long-spined sea urchin species *D. antillarum* was the most important benthic herbivore on Caribbean reefs until 1983, when it suffered mass mortality of on average 98% by a Caribbean wide pathogen (Lessios *et al*., 2001). Post-mortality scarcity resulted in increased turf/macro-algae abundance. This condition in turn decreased coral reef cover since algae compete for space with juvenile corals (Mubmy *et al.,* 2007). *D. antillarum* recovery is key to reducing macroalgal cover and increasing abundance of juvenile corals on Caribbean reefs (Edmunds & Carpenter, 2001). In the past couple of decades some natural recovery of *D. antillarum* has been observed but this has been slow and variable (Alice Rogers & Lorenzen, 2016).

Recent efforts to increase *D.antillarum* abundance focus on increasing local abundance artificially by either raising them in laboratory settings and subsequently seeding them on reefs (Williams, 2016), or by creating favorable conditions for larvae settlement with artificial reef structures (Hylkema *et al*., 2020), executed on Saba and St. Eustatius. While these and similar restocking practices already have the potential to relieve the species from some hurdles for recovery, identifying more reasons for their natural absence can aid in finding solutions for assisted recovery. Bottlenecks for recovery exist in various life stages of the sea urchin, such as in larval supply as a result of too few adults, (Lessios, 2005), in settlement and post-settlement survival due to a lack of suitable locations and high predation pressure (Rogers & Lorenzen, 2008), or even pathogens remaining in water (Lessios, 2005).

In the last decade, a new potential problem adds to the causes of the slow recruitment of the sea urchins on reefs in the Caribbean: the occurrence of drifting *Sargassum* floats. While occurrence of these floats, otherwise known as 'golden tides', along coastlines is common for most Caribbean island nations (Louime, Fortune & Gervais, 2017), during the last decade the occurrence of these mats along Caribbean coastlines has increased and concerns regarding their environmental and economic impact have risen (Putman *et al*., 2018; van Tussenbroek *et al*., 2017; Wang & Hu, 2017). Once these golden tides wash ashore, they decompose into murky '*Sargassum* brown tides'. Besides the obvious economic impact that beached decaying *Sargassum* floats have via the appeal for tourism (Putman *et al*., 2018), the effects extend to reduction in light, oxygen and pH which ultimately result in changes in community structure and the decrease of healthy coastal habitats (Cabanillas-Teran *et al*., 2019; van Tussenbroek *et al*., 2017). D. *antillarum* herbivory specifically was found to be negatively impacted by these *Sargassum* blooms through organic matter inputs coupled with hypoxia that lead to detrimental modification of natural algal resources (Cabanillas-Teran *et al*., 2019). However, the effect of *Sargassum* floats on recruitment of *D. antillarum* has not been investigated to date. Seasonal occurrence of these floats throughout the Caribbean is variable, but the greatest influx of these floats for the leeward islands tends to happen from spring, through summer and into the fall months (Hinds *et al*., 2016), with a peak in summer (Brooks *et al*., 2018). The reproductive periodicity of *D. antillarum* largely coincides with this peak in *Sargassum* influx. With the recent increase in abundance of *Sargassum* floats, the question arises if *D. antillarum* larvae are increasingly attracted to chemical cues from these floats instead of reef structures and that their final fate might then be to be beached and die, never to be contributing to the recovery of *D. antillarum* populations.

Reason to think this phenomena might be happening are results of investigation into settlement cues for echinoderm larvae. Cues hypothesized to induce settlement of larvae are biochemicals from adult populations (Hunte and Younglao, 1988). Others suggested that *D. antillarum* is attracted to cues that arise from nutrients or algae (Alice Rogers & Lorenzen, 2016). For the urchin species *S. droebachiensis* and *S. purpuratus* macro-algae were found to have no effect on metamorphosis but instead the

bacteria on the algae were found to have a positive effect (Pearce & Scheibling, 1991; Dworjanyn & Pirozzi, 2008). Similarly, for the species *P. lividus* a positive effect of macro-algae was found but it could not be excluded that this effect was due to the biofilm on the algae (Castilla-Gavilán *et al*., 2018; Rial *et al*., 2018). Bak (1985) concluded that for *D. antillarum* the absence of turf- and macro-algae was important for settlement, but found that they preferred to not settle on freshly placed and clean panels indicating a preference for slightly older biofilm coated panels. While the exact source for the settlement cues is debatable, laboratory experiments with a handful of algae substrates hinted that *Sargassum fluitans*, or its biofilm, had settlement inducing properties for *D. antillarum* (van Nimwegen, 2021). Not surprisingly though, as in the open ocean this algae functions as a suitable spawning and nursery area or habitat for numerous invertebrates, fish, turtles and birds (van Tussenbroek *et al*., 2017; Laffoley *et al*., 2011). Contrary to this finding Thabard *et al*. (2011) found that extracted molecules produced both by the alga *Sargassum polyceratium* and its associated biofilm, inhibited *D. antillarum* metamorphosis from a concentration of 5 µg/ml onward.

While laboratory studies might indicate the settlement inducing capacity of *Sargassum* spp. or even the opposite, the question whether floating *Sargassum* rafts actually affect settlement of *D. antillarum* in situ, remains unanswered. For that phenomena to happen, *D. antillarum* larvae and *Sargassum* floats need to meet. The free floating, larval stage of the sea urchin allows for their occurrence at varying water depths. Williams *et al*. (2011) found an optimal settlement depth for this species at 9 meter, and lower settlement at the shallower locations. While most *Sargassum* drifts at the surface, wind action can force the floats to sink (Johnson & Richardson, 1977). Settlement densities on *Sargassum* can therefore be hypothesized to vary for different depths. Here we investigate to what extend the phenomena of settlement on *Sargassum* patches might be happening at two different depths and discuss if it is on a scale that might have an impact on *D. antillarum* recruitment. Additionally, a pilot investigation into the species composition of drifting *Sargassum* patches showed a prominent amount of *D. antillarum* predators in samples taken. Because this could influence survival of young settlers the possible effect is accounted for in the research design. If *D. antillarum* are found to be attracted to the *Sargassum* floats in obvious abundances, this can add to the explanation of their limited recovery rates.

1.2 Research questions

The forenamed issue can be divided into the following research question and sub questions.

> To what extend do *D. antillarum* settle on *Sargassum* and is there an influence of location in the water column on settlement?
>
> - What is the density of *D. antillarum* settlement on *Sargassum* at the surface and on submerged *Sargassum*?
> - Is potential predation on *D. antillarum* settlers of influence on post settlement survival in naturally occurring *Sargassum*?

1. Methods

2.1 Research location and timeframe

The experiment was executed along the Caribbean island of Saba. Previous investigations into *D. antillarum* settlement densities around Saba in 2020 by Mareike de Bruyn and Alwin Hylkema revealed that settlement densities were highest on the west side of the island at the locations named ‘Ladder bay’ and ‘Torrents point’ (Fig. 1). Ladder bay features a flat sandy area between patchy reefs which facilitates the placement of an experimental setup. Previous data of 2020 showed that settlement on Saba starts in April and continues until November and no settlement had been observed from December till March. The optimum conditions for settlement of *D. antillarum* on Saba were in May

and June. While deployment of the experiment earlier in the year is thus advisable, the experiment was deployed at Ladder Bay from the 29th of July till the 24th of August 2021.

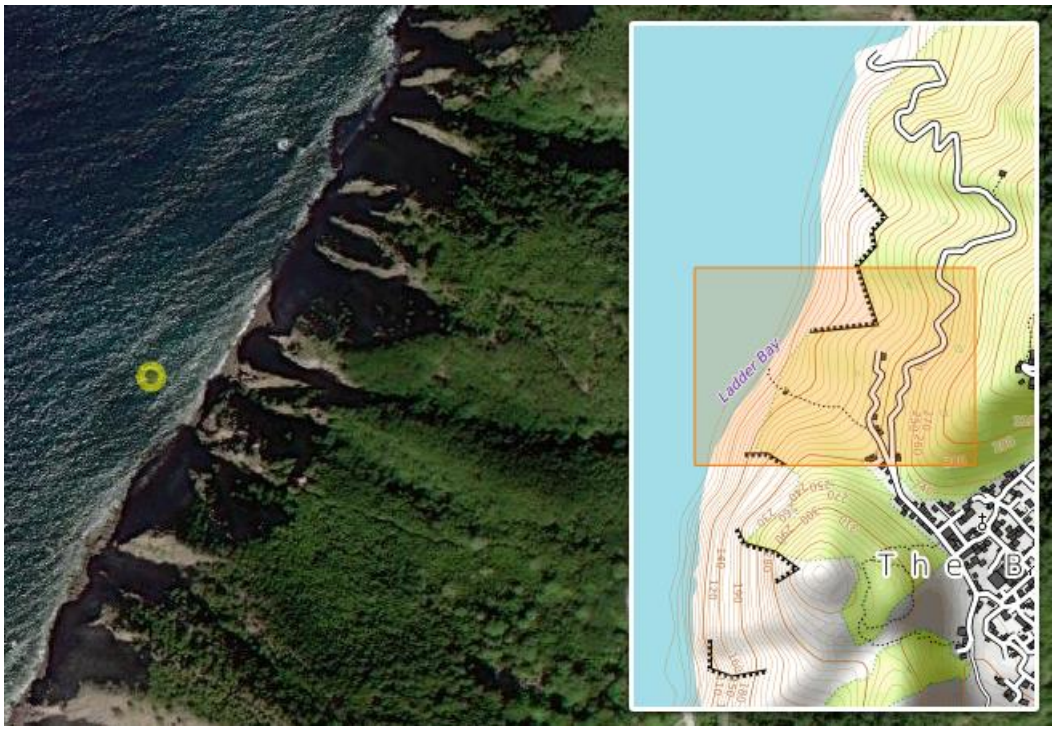


Figure 1: The location at Ladder bay with repeats of the experiment. Each repeat in turn has a set-up as indicated in figure 3. The set-up furthermore follows the design as depicted in appendix II, figure 3.

To answer the question of differential settlement density as a function of depth and simultaneously figure if there is an effect of present predators, a 2x3 research design has been used. Three different treatments at two depths with four replicates each amounted to 24 experimental units. The first treatment is a positive control group to compare settlement densities with. The control group consists of frames filled with strings with so-called *bio balls,* balls that whose surface area is optimized to facilitate maximum biofilm formation for fish ponds and aquaculture and which have shown to function as useful echinoderm settler collectors (Balsalobre *et al*., 2016). Each unit in this treatment consists of a 25 x 25 cm (2 inch diameter) PVC frame filled with 2 strings of 65 bioballs each, creating a surface area of approximately 0.85 m^2 per unit. This surface area for an experimental unit was decided upon because the corresponding volume of *Sargassum* could consistently be taken from a drifting patch with a 24 inch sieve. The area of a volume was measured using Fiji (imageJ) version 2.1.0 according to "*Protocol for area measurement using (Fiji)ImageJ v2.1.0*" (Appendix 0). The second and third treatment had identical frames filled with an equivalent of respectively rinsed and unrinsed *Sargassum* taken from a big drifting patch *"Protocol collection Sargassum, deployment of experiment and processing"* (Appendix I).

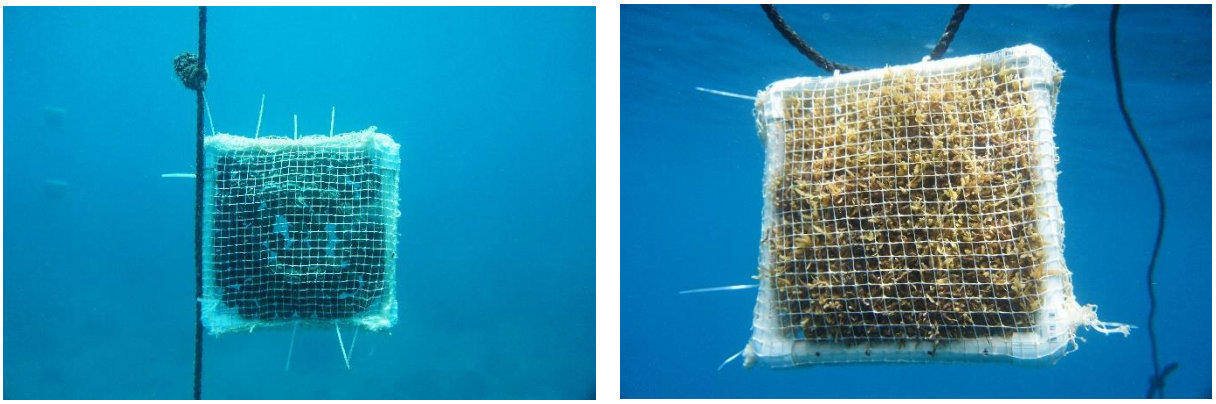


Figure 2: view of respectively the bioballs and Sargassum in an experimental unit. The sample with the bioballs in the figure is one at 7 meters below the surface while the Sargassum sample is one at the surface.

Experimental units were hung at the surface and at a depth of 7 meter (± 0.5 m) at the dedicated location at ladder bay. Treatment and secondary treatment by location in the water column resulted in 6 different partitions (Table 1).

Treatment name	*Treatment ID*
Bioballs at surface	Bio_Opp 4 replicates: (1_1 | 1_2 | 2_1| 2_2)
Bioballs subsurface	Bio_Sub "
Rinsed Sargassum at surface	Rins_Opp "
Rinsed Sargassum subsurface	Rins_Sub "
Unrinsed Sargassum at surface	Unrins_Opp "
Unrinsed Sargassum subsurface	unrins_Sub "

Each of these 6 secondary treatments received 2 spots to hang the replicates according to figure 3. At each of 6 spots two ropes are anchored to the same anchor point, usually interlinked concrete blocks. Each rope received 2 replicates of a treatment. The surface rope has 2 of the 4 replicates of a surface treatment and the subsurface rope received 2 of the 4 replicates of a subsurface treatment. The remaining four replicates of these two secondary treatments would then be hung at a second spot. Similarly, the two other treatments got two dedicated spots each.

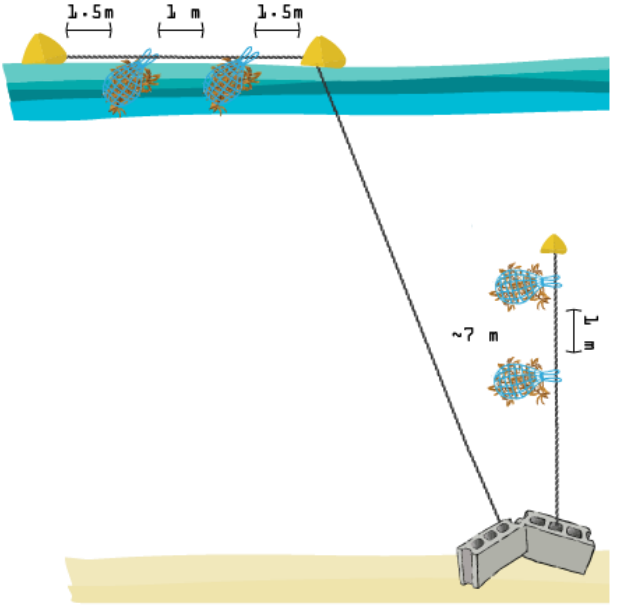


Figure 3: Six spots along ladder bay will have the set up as depicted. Per treatment (rinsed/un-rinsed/Bioballs), two spots were occupied. Per spot, two frames were hung at a surface line, spaced 1 meter apart, and two were hung at a sub-surface rope approximately 7 meters depth, spaced 1 meter apart.

2.3 Deployment , data collection & analysis

Apart from one rope all sub-surface ropes had been in the water for some time and *D. antillarum* had already migrated on the ropes. Therefor ropes were inspected before deployment and *D. antillarum* were translocated to nearby reefs before experimental units were placed. Surface ropes were not inspected because they had not been in the water for longer than 5 days. On the deployment date a *Sargassum* patch was visited and a volume sufficient to fill 26 PVC frames was put in a cooler. 16 volumes were used for the experiment and 10 volumes were taken and searched for inhabiting species according to *"Protocol collection Sargassum, deployment of experiment and processing"* (Appendix I). Three *Sargassum* types are commonly found in the region and for 4 of the 10 volumes the length of these different species and types of most common *Sargassum* were measured (Appendix I), to figure species ratios of *Sargassum* used in the experiment. After the four week duration of the experiment, experimental units were taken on land and searched for *D. antillarum* and other inhabiting species according to *"Protocol analysis D. antillarum settlement Sargassum"* (Appendix II). *D. antillarum* were collected and photographed for length measurements with Fiji (ImageJ) version 2.1.0 following "*D. antillarum measurement protocol ImageJ v1.0"*.

Length and count data of *D. antillarum* were analyzed and visualized using R, RStudio version 4.0.4. Data from a study on *D. antillarum* settlement on *Bioballs* earlier in 2021, was used to filter between settlers and older *D. antillarum* (van de Pas, 2021). There freshly placed bioball streamers are hypothesized to mainly attract settlers as older *D. antillarum* are prevented from climbing the thin fishing line that attaches the streamers to reefs. In the calculation of the mean, data for both monitoring months were pooled because a similar density pattern could be recognized (Fig. 4) and settler length is assumed to have one mean length over this period. Because of the left skew of that data, length values were natural log-transformed before a mean and standard deviation were computed. The back-transformed mean and standard deviation were used in the computation of the left bound of settler length: $exp(mean\ of\ log-transformed\ length + (sd\ of\ logtransformed\ length * 2))$ ~ 1.7. *D. antillarum* longer than this length were classified as post-settler *D. antillarum* in the analysis.

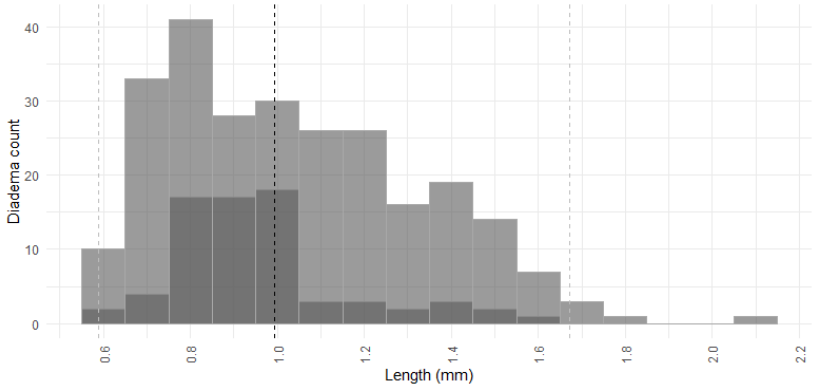


*Figure 4: Histogram of D. antillarum counts per length of data from May (dark grey) and June (light grey) in 2021, with pooled mean (black dashed line) and 2*standard deviation (grey dashed lines).*

Filtered data was then analysed using two non-parametric kruskall wallis tests for overall treatment differences. The formula *compare_means(method="Kruskal")* was applied to the submerged treatments and treatments at the surface. This was followed by a within treatment Wilcoxon-Mann-Whitney-U ranked sum test using the formula *Wilcoxon.test(paired=F). However,* because of the limited amount of *D. antillarum* found to underpin statistical analyses, hypothesis were formulated about the occurrences in the different treatments.

3. Results

3.1 *Sargassum* type ratios and inhabiting species

For deployment of the experiment a *Sargassum* patch was visited and 10 volumes were kept behind for sampling after deployment. In these samples a total of 3 regular crabs, 13 regular shrimp and 3 fireworms were found. No *D. antillarum* were found in these samples. While the protocol asked for directly putting samples in separate bags, this was forgotten and samples were taken from left over *Sargassum* in the coolbox after deployment of the experiment. This way, possible inhabiting species could have redistributed themselves to cooler areas in the cooler and could have escaped the *Sargassum*.

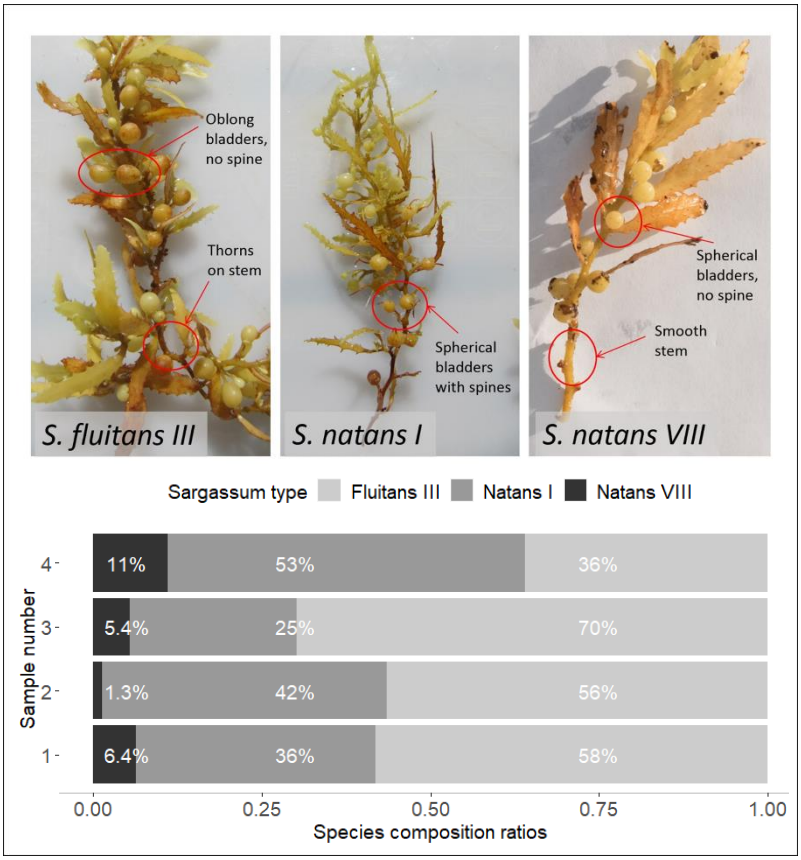


Figure 5: The three most common pelagic Sargassum morphotypes encountered around Saba: S. natans I, S. natans VIII, and S. fluitans III and their ratios in samples at time of deployment. Figure adapted from Govindarajan et al., (2019).

Stalk length measurements were used as a proxy for relative surface area of the *Sargassum* in a sample. *S. fluitans III* revealed to be most abundant in the patch, accounting for up to 70 percent of the total stalk length in the samples (Fig.5). *S. natans VIII* was least abundant. It has however to be noted that leaf count and area differs considerably between the different *Sargassum* species and types and that length is a mere approximation for their ratios within a sample.

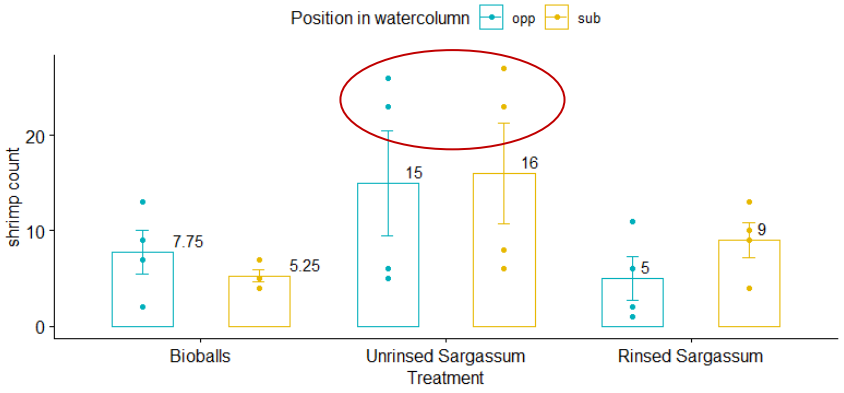


Figure 6: Barplots of mean settler density per treatment and secondary treatment, with standard error for the mean.

No obvious amount of inhabiting creatures was found in the samples at time of deployment. However after the experiment, samples were searched for inhabitants again and much more creatures were encountered. Especially the amount of shrimp other than praying mantis shrimp was high: 232 divided over the 24 samples. Most (~50%) of these shrimps were found in the unrinsed *Sargassum*, while rinsed *Sargassum* as well as the bioball treatment both had around 25 % of the total amount of shrimp in them. Four unrinsed *Sargassum* units, 2 at the surface at separate locations and 2 sub-surface, also from a separate location each, had an average of 24.75 shrimps in them. This in contrast to the rinsed *Sargassum* units, which had on average less than 10 shrimps in them, both sub-surface and at the surface, similar to the amount found in the bioball units that had no species on

them to begin with (Fig. 6) This obvious difference, however not significant, indicates that rinsing the *Sargassum* changes the abundance of creatures entering the experiment. Rinsing thus seems to be a viable method to have inhabitant presence mainly consist of influx by new creatures.

3.2 *D. antillarum* count and length distribution

During the 26 day experiment a total of 71 *D. antillarum* were found (one photo for length analysis was missing and length could not be computed for this *D. antillarum*). Length ranges and found amounts of *D. antillarum* were quite variable per treatment. Most *D. antillarum* were found in the unrinsed submerged *Sargassum* and biggest size ranges were found in the unrinsed *Sargassum* treatment (Table 1).

Table 1: Sizes, size ranges (both in mm) and amount of found D. antillarum per treatment. Of the D. antillarum found per treatment a certain amount can be classified as settler. The cut-off length for being classified as a settler is 1.67 mm.

Treatment	*Min (mm)*	*Max (mm)*	*Range width*	*Total D. antillarum count*	*Total settler count*	*Mean settlement density (m^{-2})*
Bio_Opp	0.764	0.864	0.100	2	2	0.58
Bio_Sub	0.525	3.355	2.830	9	8	2.32
Rins_Opp	2.232	3.918	1.686	2	0	0.00
Rins_Sub	2.341	3.659	1.318	6	0	0.00
Unrins_Opp	1.401	5.714	4.313	5	1	0.30
Unrins_Sub	0.905	4.629	3.724	46	11	3.60

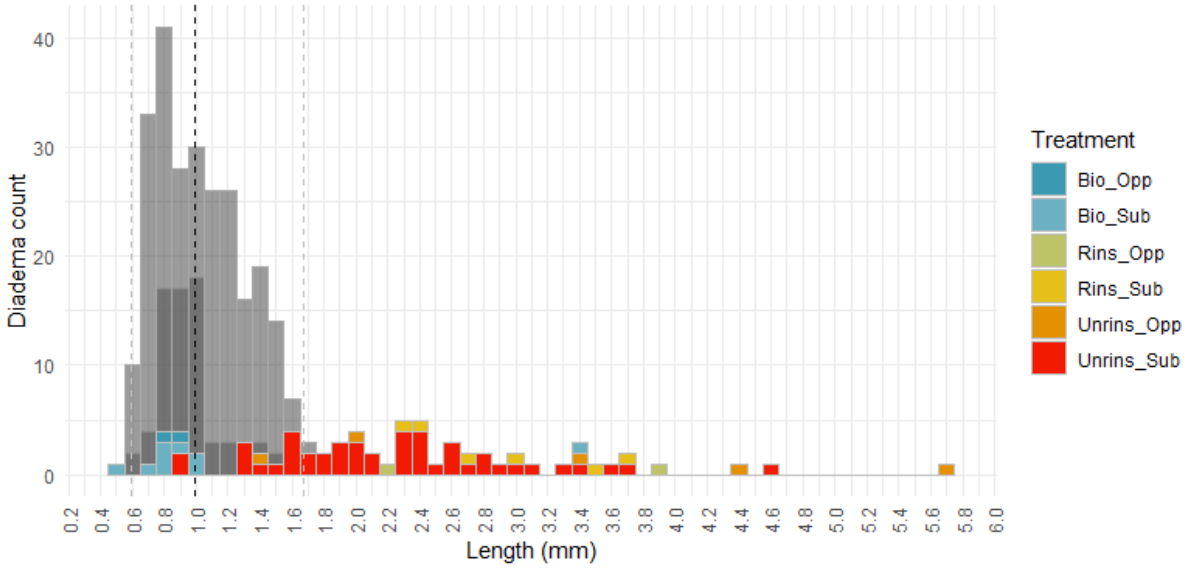


Figure 7: D. antillarum counts per length and treatment. Grey bars in background reflect the densities in the settlement in the bioballs experiment from June (darkgrey) and July (lightgrey). Counts for these months are high because more experimental units were searched for D. antillarum. Mean length for cumulative counts for these months is indicated with black dotted line and the grey dotted lines indicate the distance of 2 standard deviation away from the mean. D. antillarum with a length below the upper boundary of that range classify as settlers in our experment.*

On the basis of settlement counts on bioballs from June and July earlier in the year, a cut of value for classifying as a settler of approximately 1.7 mm was constructed. Although these bioballs were hanging at a different depth, had only 30 balls per string (with a total surface area of 0.2 m^2) and hang earlier in the year, this data gives a clear indication of the length distribution of settlers over a four week period. These bioballs also hung in the water for a month and are assumed to foremost attract settlers. Most *D. antillarum* settlers were found in July during that experiment. Here, when extrapolating to a surface area of 1 squared meter, a mean settlement density of 41.1 m^{-2} was found in June and a mean settlement density of 143 m^{-2} was found in July. These amounts are contrasting the current study where on the bioballs at the surface a mean density of 0.58 m^{-2} was found and on the submerged bioballs a mean density of 2.32 m^{-2} was found (Table 1, Fig. 8). The bioball treatments had the highest relative amount of settlers on them compared to the overall *D. antillarum* count, followed by the unrinsed treatment (Table 1, Fig. 7). Highest absolute settler densities were found in the unrinsed submerged treatment. However, of the total *D. antillarum* found in the *Sargassum* treatments, only 20 % could be classified as actual settlers. This percentage is furthermore mainly driven by one observation in which 27 *D. antillarum* were found of which 10 *D. antillarum* were settlers, accounting for 17% of the settlers found in *Sargassum* treatments. The rinsed *Sargassum* treatment did not attract settlers.

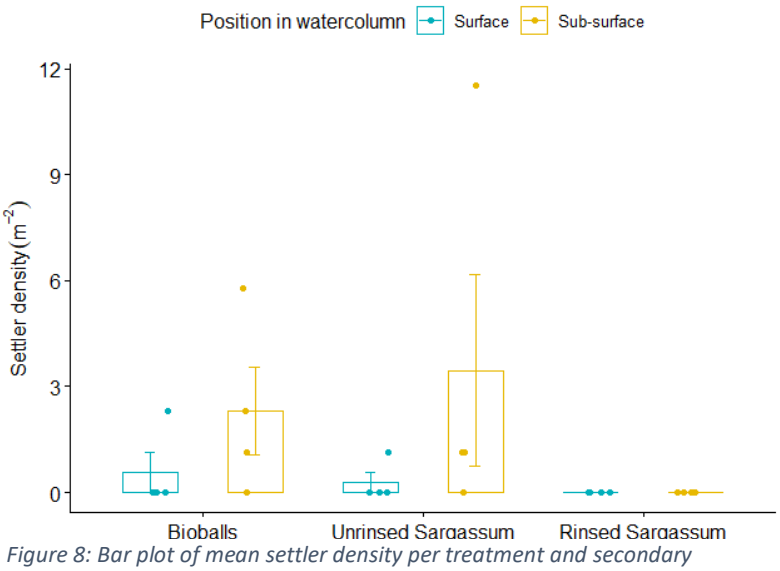


Figure 8: Bar plot of mean settler density per treatment and secondary treatment, with standard error for the mean.

When simply looking at occurrences of the different classifications of the life-stages for the different treatments we find the following. The bioballs mainly attracted settlers, 2 at the surface 8 sub-surface, and 1 *D. antillarum* supposedly migrated into the experimental unit. In the rinsed *Sargassum* only post-settlement *D. antillarum* were found, 2 were found in the same surface unit, and 6 were found in 3 sub-surface units which each had 2 of these *D. antillarum.* In 11 of the 16 experimental units *D. antillarum* were found. In only 4 of these 11 units *D. antillarum* could be classified as settlers.

In the unrinsed *Sargassum* a total of 13 settlers was found. 10 of these were found in a submerged unit that had 27 *D. antillarum* in total in them. 1 settler was found in a surface unit together with a post-settler and the other 2 settlers were found in submerged units with 9 and no post-settlement *D. antillarum* respecitvely. The remaining submerged experimental unit also had a relatively high amount of post-settlement *D. antillarum* (9), but no settlers. The rinsed *Sargassum* treatment did not have settlers but did have 3 experimental units with 2 post-settlement *D. antillarum* per unit each.

The big amount of *D. antillarum* found in that one submerged unit with 27 urchins coincides with the high amount of shrimp. However, this was not necessarily the case for other units were many shrimp were found. Overall, no correlation between the amount of predators and the *D. antillarum* counts has been found. Finally, kruskall wallis rank sum tests between treatments revealed that no significant difference in medians could be detected between sub-surface treatments as well as between surface

treatments (Surface treatments: chi-squared = 1.1136, df = 2, p-value = 0.573 & Sub-surface treatments: chi-squared = 4.8209, df = 2, p-value = 0.0897). Similarly, Wilcoxon rank sum tests revealed that no significant difference between medians could be detected within treatments (Bioballs: W= 4, p-value = 0.2784, Unrinsed *Sargassum*: W=3.5, p-value = 0.2059, Rinsed *Sargassum*: no settlers).

4. Discussion

The amount of data collected did not suffice to answer the research question in any statistically significant manner. However, the observations gave reason to formulate hypothesis from the findings. From the Bioball treatment one can hypothesize that there is an effect of depth on settlement. Here only 1 post-settlement *D. antillarum* was observed, which must have migrated from the rope into the experimental unit. Higher settlement densities were found at 7 meters depth. This could have to do with the disturbance by waves at the surface, but a reduced abundance at the surface was not obvious when looking at the combined abundances of species other than *D. antillarum*. This hints that depth is likely the factor mostly affecting the observation since apart from a difference in wave action no variables other than depth obviously differed for the surface and submerged Bioball treatment.

Also for the unrinsed treatment higher settler densities were found in the submerged units, but since in the experimental unit that had the bulk of the settlers also 17 post-settlement *Diadama* were found, the observation cannot simply be explained by depth only. Conspecifics are found to have a settlement inducing effect for sea urchins (Pearce & Scheibling, 1991; Dworjanyn & Pirozzi, 2008), and could have influenced settlement densities in these experimental units. The observation that no settlers occurred in the rinsed *Sargassum* treatment that had a maximum of 2 post-settlement *D. antillarum* per experimental unit, strengthens this idea.

The one observation of the unit with 10 settlers that also had 17 post-settlement *D. antillarum* is basically driving the observed amount of settlers in *Sargassum* on its own. In other *Sargassum* units, either rinsed or unrinsed, on average more post-settlement *D. antillarum* were found than in the bioball treatment, but apart from that one observation, settler abundances did not obviously differ from the observed abundances of settlers in the bioballs.

Observed amounts of post-settlers in *Sargassum* were highest in the unrinsed submerged *Sargassum*. Reasons for this finding could be that these *D. antillarum* were able to migrate over the ropes into the experimental units of the submerged treatments. Since the submerged ropes the experimental units were tied to had *D. antillarum* urchins on them at the beginning of the experiment, some migration into the experimental units on these ropes may have occurred. For the surface treatment, the ropes where however freshly placed and had no prior ecosystem on them. Additionally *D. antillarum* had to migrate over a relatively long distance from established ecosystems on the sub-surface ropes to the experimental units at the surface making migration to these units unlikely. This is reflected in the *D. antillarum* counts of the bioball treatment. There, no found *D. antillarum* at the surface did classify as post-settler *D. antillarum*. In the sub-surface bioball treatment 1 post-settler *D. antillarum* was found which must have migrated from the rope onto the bioballs. That migration to surface units is certainly happening is reflected in the occurrence of creatures other than *D. antillarum* in these units for the bioball treatment.

While unrinsed submerged *Sargassum* had a relatively high amount of post-settlers in them, unrinsed *Sargassum* at the surface had at maximum two post-settlers in them. The same ratio was however not observed for the rinsed *Sargassum* were the amounts of post-settlers for the surface and submerged treatment did not differ obviously. If one follows this logic, the post-settlers found in the unrinsed *Sargassum* could thus be mainly urchins that remained in the *Sargassum* after being taken from

drifting *Sargassum* patch at time of deployment. The variation found for unrinsed *Sargassum* is then an artefact of sampling from a non-uniform distribution of *D. antillarum* in a drifting *Sargassum* patch. However, in a 3 week pilot from June 19th till July 9th, 15 post-settlers were found in a volume similar to that of the current experiment filled with rinsed *Sargassum*. This volume also hang from an already present rope, and since *Sargassum* was rinsed these *D. antillarum* likely migrated from the rope into the *Sargassum.* To confidently follow any of these hypotheses more experimental evidence is necessary. In any way, post-settlement *D. antillarum* seem to be attracted to the *Sargassum* used in the experiment.

An indication that *Sargassum* is in fact attracting post-settlement *D. antillarum* is the observation that there was only 1 post-settlers found in bioballs as opposed to 4 occurences where 2 post-settlement *D. antillarum* each were found in rinsed *Sargassum* under the assumption that rinsing got rid of already present *D. antillarum*. Alternatively, if post-settlement urchins did not migrate into the *Sargassum* from the ropes, they must have entered the drifting patch from an earlier life-stage, meaning that settlers are also attracted to the drifting patches.

Furthermore, *Sargassum* in submerged units had degrading over the 4 week period of the experiment, while *Sargassum* in units at the surface was actually growing. This difference is not though to have had a major effect on the *D. antillarum* densities at the two depths, as abundances of post-settlers did not differ obviously for the rinsed treatment. However, if the experiment is to be conducted again in a different month, any potential effect should be mitigated.

No *D. antillarum* were found in the 10 samples from the *Sargassum* patch used to create the experimental units and none were found in several smaller volumes taken around the island during tasks unrelated to the current experiment. From these observations, the occurrence of *D. antillarum* in *Sargassum* floats seems to be very coincidental. The absence of *D. antillarum*, but also of critters in general, from the 10 samples, can be explained by their migration to the cooler water in the coolbox before samples were put into bags. The low amount of *D. antillarum* in rinsed submerged *Sargassum* as opposed to their relative abundance in unrinsed submerged *Sargassum* at the end of the experiment points towards their presence in floating *Sargassum* rafts. That no urchins were found in the samples taken during other field tasks, could have been due to their non-uniform distribution in sargassum floats or coincidental occurrence in certain patches.

I must also add that the location the experiment was conducted at was already known to attract a relatively high abundance of settlers. This location, downstream of the main current pattern around the island and on the leeward side of the island, likely acts as a sink for the settlers. Extrapolating findings needs to be done with caution. The question whether the increased occurrence of Sargassum floats in the area may have an impact on settlement is then more a probabilistic than a quantifiable one in the sense that only every now and then a batch of larvae may end up in a patch given the right conditions. Follow-up research could link *Sargassum* float occurrence to current direction and velocity, and the float biomass to observed larval densities. This is however beyond the scope of the current project.

Conclusion & recommendation

To summarize, *D. antillarum* certainly are attracted to sargassum patches, whether as post-settler only or also as settler is however still unclear. Additionally, effect on settlement cannot solely be described to *Sargassum.* Only one observation had relatively high abundance of settlers, but that same experimental unit also had a lot of post-settlers. Cues by conspecifics could possibly play a role in increased settlement. In the current study no relation between predator occurrence and *D. antillarum*

occurrence was obvious. A large variation in *D. antillarum* abundance was found for the unrinsed submerged treatment. Two other factors apart from cues by conspecifics could have played a role here. One being different rates of migration into the experimental units from existing ecosystems on the less recently placed ropes. The other being that this variation reflects variation of *D. antillarum* abundance in drifting *Sargassum* patches and so the observed variation is an artefact of the low sample size.

To confidently distinguish signal from noise, some alterations to the research design need to be made. With a simpler , possibly unbalanced, design one can create bigger sample sizes and also introduce less variation per treatment. For instance by using strong fishing lines with cylindrical Sargassum- or bioball-filled nets attached. In such a design, surface area effect must be kept in mind however, as too small surfaces might not produce strong enough cues and density might not scale linearly with surface area. Using fishing lines instead of rope not only benefits upscaling but simultaneously tackles the problem of migration, since urchins cannot crawl on such narrow lines and so no migration is expected. This overcomes the question about cues by conspecifics, as inhibiting migration of post-settlers limits their abundance in the experimental units. A more thorough method however would be to only use rinsed *Sargassum*. With large enough sample sizes a possible effect of predator abundance on settlers can then be found by plotting the relationship between the two, which would also answer the question about the effect of predators irrespective of having unrinsed *Sargassum* as a treatment. Next to that, *D. antillarum* presence in naturally occurring drifting *Sargassum* patches needs to be looked into by sampling more patches in a standardized manner throughout the season.

While during the current study small differences in settlement between treatments were expected because of lower settlement rates later in the season, the low sample sizes and 0-occurrences combined resulted in too little information present. This could be overcome by conducting the experiment earlier in the season around the peak if May/June when settlement rates are higher. With higher amounts of settlement, means or medians per treatment would be more separated and a more decisive answer could be formulated.

Finally, while the difference in freshness of the *Sargassum* over the time is thought to have had no serious effect, it is wise that a similar experiment should minimize any potential effect the degradation can have by shortening the duration of the experiment.

References

Bak, R. P. M. (1985, May). Recruitment patterns and mass mortalities in the sea urchin *D. antillarum* antillarum*. Proc. 5th Int. Coral Reef Congress 5,* 267-272.

Balsalobre, M., Wangensteen, O. S., Palacín, C., Clemente, S., & Hernández, J. C. (2016). Efficiency of artificial collectors for quantitative assessment of sea urchin settlement rates. *Scientia Marina*, *80*(2), 207-216. https://doi.org/10.3989/scimar.04252.13A

Brooks, M. T., Coles, V. J., Hood, R. R., & Gower, J. F. R. (2018). Factors controlling the seasonal distribution of pelagic Sargassum. *Marine Ecology Progress Series*, *599*, 1–18. https://doi.org/10.3354/meps12646

Cabanillas-Teran, N., Hernandez-Arana, H. A., Ruiz-Zarate, M. A., Vega-Zepeda, A., & Sanchez-Gonzalez, A. (2019). Sargassum blooms in the Caribbean alter the trophic structure of the sea urchin *D. antillarum* antillarum. *PeerJ*, *2019*(8). https://doi.org/10.7717/peerj.7589

Castilla-Gavilán, M., Turpin, V., Buzin, F., Cognie, B., & Decottignies, P. (2018). Optimizing metamorphosis in Paracentrotus lividus aquaculture using alternative macroalgae species to Corallina sp. *Aquaculture International*, *26*(6), 1511–1518. https://doi.org/10.1007/s10499-018-0305-8

Dworjanyn, S. A., & Pirozzi, I. (2008). Induction of settlement in the sea urchin Tripneustes gratilla by macroalgae, biofilms and conspecifics: A role for bacteria? *Aquaculture*, *274*(2–4), 268–274. https://doi.org/10.1016/j.aquaculture.2007.11.030

Edmunds, P. J., & Carpenter, R. C. (2001). Recovery of *D. antillarum* antillarum reduces macroalgal cover and increases abundance of juvenile corals on a Caribbean reef. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(9), 5067–5071. https://doi.org/10.1073/pnas.071524598

Louime, C., Fortune, J., & Gervais, G. (2017). Sargassum invasion of coastal environments: a growing concern. American Journal of Environmental Sciences, 13(1), 58-64 https://doi.org/10.3844/ajessp.2017.58.64

Hinds, C., Oxenford, H., Cumberbatch, J., Fardin, F., Doyle, E., & Cashman, A. (2016). Golden Tides: Management Best Practices for Influxes of Sargassum in the Caribbean with a focus on clean-up. Retrieved June 7, 2021, from https://www.openchannels.org/sites/default/files/literature/CERMES%20Sargassum%20Management%20Brief%20%20-%20August%2024%202016.pdf

Hunte, W., & Younglao, D. (1988). Recruitment and population recovery of Diadema antillarum(Echinodermata; Echinoidea) in Barbados. *Marine ecology progress series,* 45(1), 109-119.

Hylkema, A., Debrot, A. O., Osinga, R., Bron, P. S., Heesink, D. B., Izioka, A. K., Reid, C. B., Rippen, J. C., Treibitz, T., Yuval, M., & Murk, A. J. (2020). Fish assemblages of three common artificial reef designs during early colonization. *Ecological Engineering*, *157*(August), 105994. https://doi.org/10.1016/j.ecoleng.2020.105994

Johnson, D. L., & Richardson, P. L. (1977). On the wind-induced sinking of Sargassum. *Journal of Experimental Marine Biology and Ecology*, *28*(3), 255–267. https://doi.org/10.1016/0022-0981(77)90095-8

Lessios, H. A. (1988). Population dynamics of *D. antillarum* antillarum (Echinodermata: Echinoidea)

following mass mortality in Panamá. *Marine Biology*, *99*(4), 515–526. https://doi.org/10.1007/BF00392559

Lessios, H. A., Garrido, M. J., & Kessing, B. D. (2001). Demographic history of *D. antillarum* antillarum, a keystone herbivore on Caribbean reefs. *Proceedings of the Royal Society B: Biological Sciences*, *268*(1483), 2347–2353. https://doi.org/10.1098/rspb.2001.1806

Mumby, P. J., Hastings, A., & Edwards, H. J. (2007). Thresholds and the resilience of Caribbean coral reefs. *Nature*, 450(7166), 98-101. https://doi.org/10.1038/nature06252

Pearce, C. M., & Scheibling, R. E. (1991). Effect of macroalgae, microbial films, and conspecifics on the induction of metamorphosis of the green sea urchin Strongylocentrotus droebachiensis (Müller). *Journal of Experimental Marine Biology and Ecology*, *147*(2), 147–162. https://doi.org/10.1016/0022-0981(91)90179-Z

Putman, N. F., Goni, G. J., Gramer, L. J., Hu, C., Johns, E. M., Trinanes, J., & Wang, M. (2018). Simulating transport pathways of pelagic Sargassum from the Equatorial Atlantic into the Caribbean Sea. *Progress in Oceanography*, *165*, 205–214. https://doi.org/10.1016/j.pocean.2018.06.009

Rial, D., Rial, P., Casal, A., Costoya, N., & Costas, D. (2018). Induction of settlement, growth and survival of juveniles of Paracentrotus lividus. *Aquaculture*, *483*, 16–20. https://doi.org/10.1016/j.aquaculture.2017.10.005

Rogers A, Lorenzen K (2008) Recovery of *D. antillarum* antillarum and the potential for active rebuilding measures: modeling population dynamics. In: *Proc 11th Int Coral Reef Symp*., *2*(July), 961–966

Rogers, Alice, & Lorenzen, K. (2016). Does slow and variable recovery of *D. antillarum* antillarum on caribbean fore-reefs reflect density-dependent habitat selection? *Frontiers in Marine Science*, *3*(May), 1–10. https://doi.org/10.3389/fmars.2016.00063

Thabard, M., Gros, O., Hellio, C., & Maréchal, J.-P. (2011). Sargassum polyceratium (Phaeophyceae, Fucaceae) surface molecule activity towards fouling organisms and embryonic development of benthic species. Botanica Marina, 54(2), 147–157. https://doi.org/10.1515/BOT.2011.014

Van de Pas, E. (2021), unpublished project internship VHL

Van Nimwegen, F. (2021), The effect of biochemical cues on the settlement of larvae of the sea urchin Diadema antillarum, unpublished master thesis VHL

van Tussenbroek, B. I., Hernández Arana, H. A., Rodríguez-Martínez, R. E., Espinoza-Avalos, J., Canizales-Flores, H. M., González-Godoy, C. E., Barba-Santos, M. G., Vega-Zepeda, A., & Collado-Vides, L. (2017). Severe impacts of brown tides caused by Sargassum spp. on near-shore Caribbean seagrass communities. *Marine Pollution Bulletin*, *122*(1–2), 272–281. https://doi.org/10.1016/j.marpolbul.2017.06.057

Vermeij, M. J. A., Debrot, A. O., Van Der Hal, N., Bakker, J., & Bak, R. P. M. (2010). Increased recruitment rates indicate recovering populations of the sea urchin *D. antillarum* antillarum on Curaçao. *Bulletin of Marine Science*, *86*(3), 719–725.

Wang, M., & Hu, C. (2017). Predicting Sargassum blooms in the Caribbean Sea from MODIS observations. *Geophysical Research Letters*, *44*(7), 3265–3273. https://doi.org/10.1002/2017GL072932

Williams, S. M. (2016). *Seeding reefs with D. antillarum antillarum to enhance coral recovery in Puerto Rico* (Issue August).

Williams, S. M., García-Sais, J. R., & Yoshioka, P. M. (2011). Spatial variation of *D. antillarum* antillarum settlement in La Parguera, Puerto Rico. *Bulletin of Marine Science*, *87*(3), 531–540. https://doi.org/10.5343/bms.2010.1041

APPENDIX I

Protocol for area measurement using (Fiji)ImageJ v2.1.0

By Jasper Bleijenberg, July 2021

First before using the program, make sure that you are using clear and sharp pictures for the analysis. Besides the clear and sharp pictures, it is important to standardize your setup to make sure you get the same light with every measurement. And there must be a ruler off some kind on the picture to make sure you can set the scale.

Steps I took to accomplish this were:
1. Use a stable and flat underground
2. Draw a boundary box on a sheet of waterresistant paper to place the leaves in. The known lengths of the box will function as a ruler.
3. use 3 lights in a triangular fashion (Figure 1) to minimize shadows as these will make the automatic process unable to execute properly.
4. Put camera on tripod above setup. Make sure that the whole boundary box of the sheet is in the frame and in focus (Figure 2).
5. Always use manual mode to make sure that camera settings are the same.
6. To take picture, place leaves and stalks on the paper and place sheet of glass or acrylic over them to press them down.

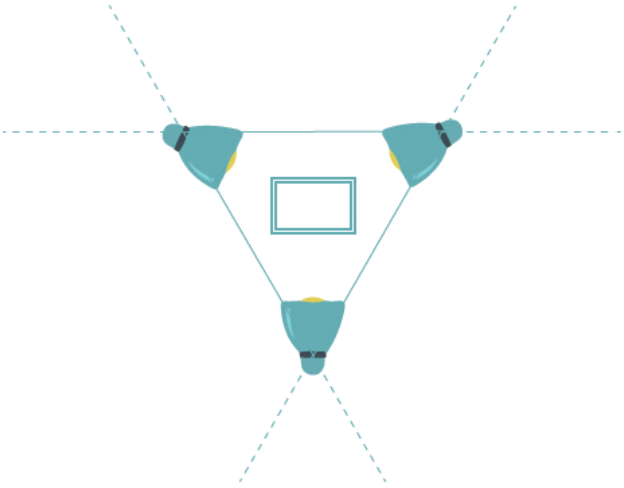


Figure 1: Place lamps in a triangular fashion around object to photograph

Figure 2: Example of image taken for analysis.

Steps in the image analysis software are:
1. Open the opensource program ImageJ v2.1.0.
2. Open the picture you want to analyze.
3. Use the rectangular selection tool and select the area you want to measure from, the bounding box.
3. In case of a shadow gradient over the image, cut the image in smaller pieces and analyze separately. Make sure to cut every image exactly the same way using the ROI (Region Of Interest) manager into pieces of the same area so that you can use the same scale in every image.
4. Adjust the type of picture, so you can adjust the threshold; Image > Type > 8-Bit. This step will convert your picture in a greyscale picture.
5. Now the scale has to be set. Use the straight selection tool and select a side of the bounding box. Now go to analyse > set scale. Now fill in its length in the box 'Known distance' and press OK. Note

down the amount of pixels that are given for the length inserted. With the next image you can use these values directly instead of using the straight selection tool first.
6. Now make two duplicates of your selected picture by pressing ‘Ctrl + Shift + D’ twice.

The first steps of this protocol were the fundament the measurement. In the next steps there is an explanation how to properly use the threshold. The rough edges of the leaves are sometimes hard to select properly, however these should be considered in the analysis.
7. To select the area you want to measure go to Image > Adjust > Threshold. Set the top bar on ‘0’ and slide the lower bar to the left, stop sliding when the whole surface area of the *Sargassum* is red 20 over 110 worked form me. Now press Apply to make a mask.
8. Sometimes the darker borders get included in the process, to remove these select them and delete from mask.
9. To measure the surface area of the *Sargassum*, go to Analyse > Analyse particles. Now make sure that the settings are just like in table 1. When the settings are correct press OK. Two windows should pop up. One with the results and one with the outlines. In the window with the outlines, you are able the control which numbers in the results window are the surface area of the measurement. Save the results and write down the surface area of the *Sargassum* as a back-up.

Table 1: Settings of analyze particles tool

Analyse particles settings:
Size cm^2 → 0.005-1.00
Show → Outlines
Circularity → Default
Tick: “include holes”, “display results”

10. The saved results can now be analyzed in R studio.

Appendix II

Protocol collection *Sargassum*, deployment of experiment and processing

By Jasper Bleijenberg, July 2021

Summary

On the day there are 3 tasks, first we **prepare net bags for deployment** later on. For this, a *Sargassum* patch will be visited and an amount of *Sargassum* enough for 26 netbags has to be put in the coolbox. 16 volumes (Fig. 1) are for the actual netbags and 10 volumes will be samples to reconstruct the composition of *Sargassum* species in the patch and get an idea of the creatures that inhabit the *Sargassum*. 8 volumes of *Sargassum* need to be rinsed and 16 bags need to be filled with *Sargassum*, 8 unrinsed and 8 rinsed samples.

Then for **the actual deployment**, these 16 bags and 8 bags with bioballs that equal the same amount of surface area as the *Sargassum* ones need to be hung at the ropes at ladderbay. Four netbags per spot; two at the surface, and two at 7-ish meter at the ropes with the subsurface buoy, filled with the different treatments (Fig. 2). All netbags need to be 1 meter separated. While there, we will inspect the ropes to tally already present *D. antillarum* and possibly photograph some.

Lastly, on shore we **process** the 10 **samples** of *Sargassum* by searching for creatures and divide the samples into species/morphologies to deduce the composition of the *Sargassum* patch we visited.

Materials	Amount
frames	6*4=24 + some extra = 28
Bioballs	
Waterproof data sheet + pencil (with eraser)	4
Tie-wraps	
Tubs/bukets	10/5
Coolbox (the big one)	
Underwater camera	
Dive computer + scuba gear + **diveknife** + datasheet with writing slate	1 set per diver
Sieve	
Marked 2-gallon ziplock bags	30
scuba tanks	6
Mesh bag	3

Preparation of bags for deployment

Collection of *Sargassum*

- Find single *Sargassum* patch big enough to fill 26 frames.
- Fill the coolbox .

Sampling

- Fill sieve to the rim with *Sargassum* (Fig. 1)
- Put this volume in a 2-gallon Ziplock bag and store in coolbox
- Repeat 10 times to have samples to search after deployment.

Rinsing and preparing *Sargassum* bags

Figure 1: Filing the sieve till the rim amounts for 1 volume. So a little less than this actually

- Put the 10 white tubs on the floor of the boat and fill with seawater strained with the sieve
- Fill sieve to the rim with *Sargassum* (Fig. 1)
- Divide in 5^{ths} and rinse each part thoroughly in a tub.
- After that put all *Sargassum* of that round in bucket with fresh seawater and rinse one final time in the bucket. (Hopefully the extra space will give some organisms the intention to swim to the bottom.)
- Put rinsed *Sargassum* in a prepared frame and close the frame with some tie-wraps. Also attach colored tie wrap to recognize that this is rinsed *Sargassum*.
- Put frame in 2-galllon ziplock bags and leave in the cooler for now
- Refresh the bucket with seawater
- Repeat this step a total of 8 times.

Preparing *Sargassum* bags without rinsing

- Fill sieve to the rim with *Sargassum*
- Put this volume of un-rinsed *Sargassum* in the prepared white frame and close the frame with some tie-wraps.
- Put frame in 2-galllon ziplock bag and put in coolbox.
- Repeat this step a total of 8 times

Deployment of bags

The 24 bags need to be hung from 12 lines at 6 spots

Every 3th spot has the same treatment

Order of treatments is **unrinsed – rinsed – bioballs**– unrinsed – etc…

We are with 4 people diving at location close to coral nursery (Fig. 3)

1 extra person aboard would be helpful

Task

- 2 persons attach frames to the surface ropes.
- At the same time, 2 persons gear up for diving and start inspecting the sub-surface ropes for already present *D. antillarum*. They also clean the rope as good as possible within 5 around minutes per rope.When ropes are cleaned, they surface.
- Now everyone gears up for diving. 3 persons each take 2 frames from a treatment.
- At the 7 m mark, the person without frames receives frames from one treatment and ties these at the rope with 1 meter in between the frames (Fig. 2)

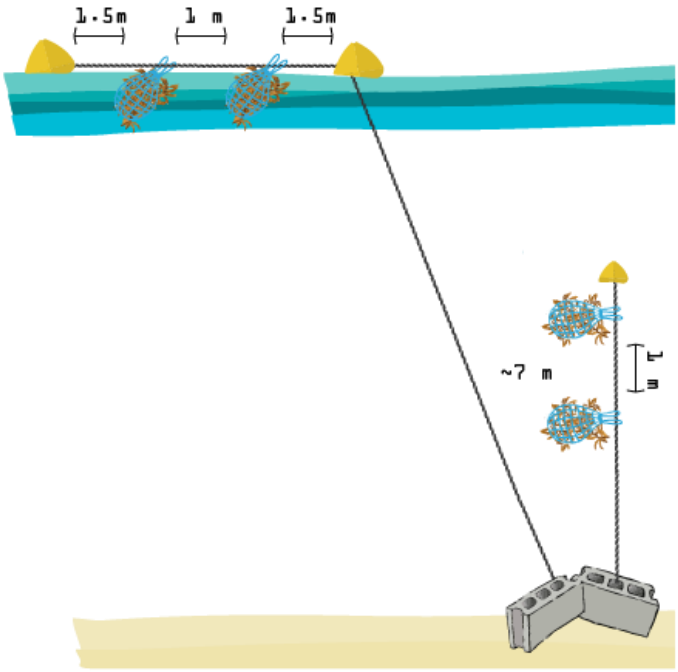


Figure 2: view of spot with particular treatment. 6 of these spots will be visited.

! You can tie a bag at the rope by twisting the rope and putting the free tie-wrap on the bag through the cavity that this creates !

- The result has to look like figure 4(A).
- Swim to next subsurface buoy and repeat but for the different treatment.
- Repeat in a second dive with the remaining 6 frames.

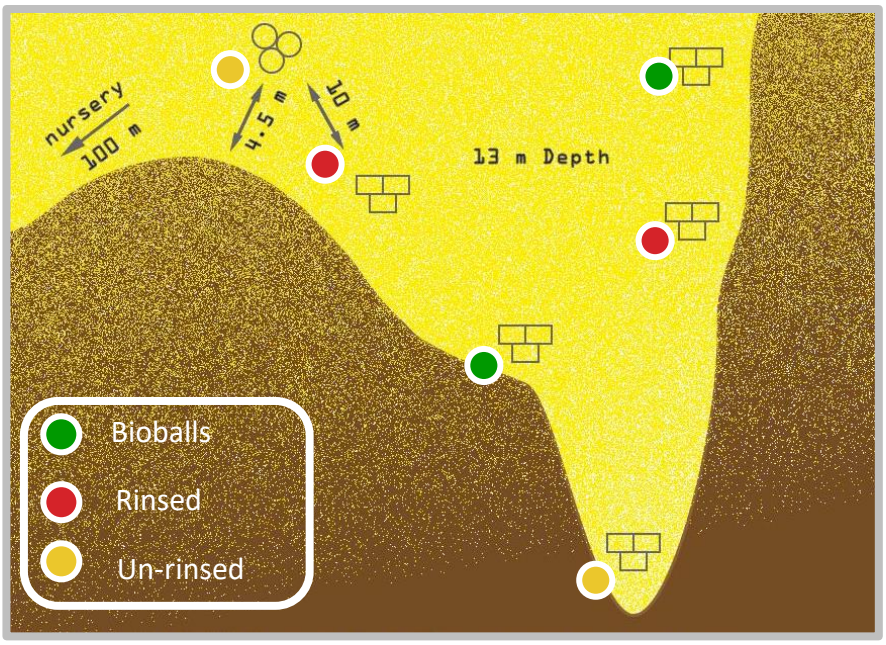


Figure 3: Schematic top view of location of experiment. Clustered rectangles represent cinderblocks while clustered circles represent location where ropes were attached to unused reefball. The location is about 100 m away from the coral nursery.

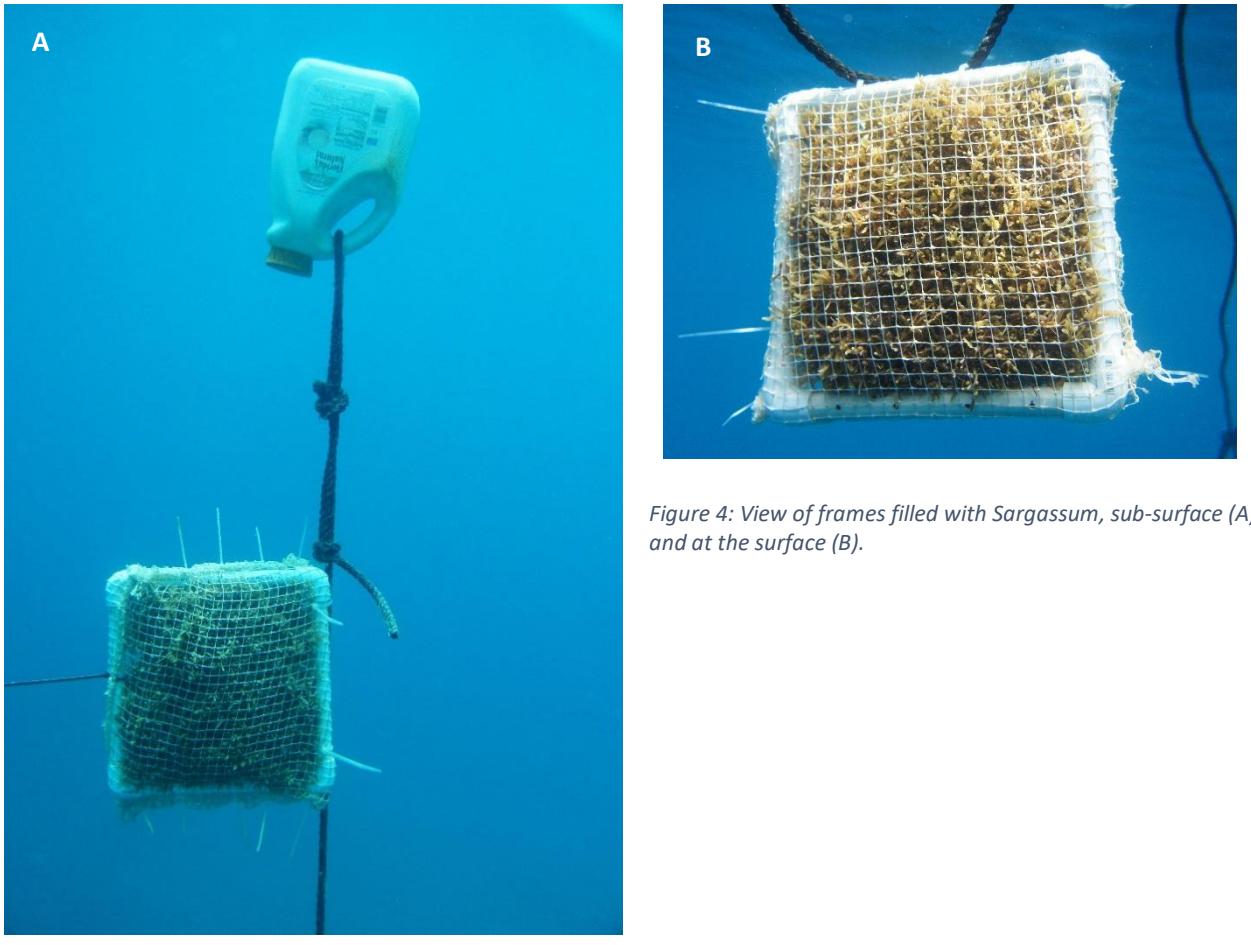


Figure 4: View of frames filled with Sargassum, sub-surface (A) and at the surface (B).

Processing *Sargassum* samples using tweaked "protocol analysis *D. antillarum* settlement collectors"

Materials
Samples collected in zip-lock bags
White tubs, containers
Analysis sheet on waterproof paper + pencil
3 buckets: one with clean, fresh sea water, one to throw away the analyzed samples and one with fresh water.
Pipettes
Sticks/skewers for sorting
Sealed mm paper
Camera
Small container (e.g. old yoghurt cup) to store *D. antillarum* after analysis
Cooler with fresh sea water
Whiteboard

Searching for creatures in the *Sargassum*

All samples are analyzed separately and as soon as possible after collection!

1. Start with the samples that were first collected.
2. Try to remove all organisms from the *Sargassum* by shaking the sample thoroughly in the zip lock bag.
3. Divide sample over 5 containers. (With fresh *Sargassum*, 5 containers is sufficient.)
3. Pour the remaining water in the bag into a container. Make sure no organisms stick in the bag.
4. Rinse the divided samples in the container thoroughly and put the *Sargassum* in the cooler with sea water. (This *Sargassum* will be identified for species composition later on)
6. Place the containers on a table and let them rest for 1 minute
7. Search the containers carefully for organisms, identify them and record them on the analysis sheet. Use the document "Identification of marine organism in *D. antillarum* settlement traps".

The following organisms must be identified:

1. *D. antillarum*
2. Other sea urchins (up to species level if possible)
3. Crabs (Spider crab/crab other)
4. Hermit crabs
5. Shrimps (Mantis shrimp/shrimp other)
6. Lobster
7. Worms (fireworm/worm other)
8. Other crustacea

D. antillarum can be as small as 1 mm, so look really carefully! Also, if you find a *D. antillarum*, you can suck it up with the pipette, but be careful, because if you wait too long with putting it in the petri dish, the *D. antillarum* will adhere to the pipette.
Living *D. antillarum* adhere quickly to the container, if you shake the container carefully you can find them easily by looking at small round, red dots that are not moving. If you

look more carefully, you can see the (striped) spines of the *D. antillarum*. Collect both living and dead *D. antillarum*.

10. We usually do not find other sea urchins, but you can always recognize *D. antillarum* because they have striped spines and are very red after settlement. They turn black within 2 weeks, so you can also find black *D. antillarum* (but these still have striped spines). If you find other sea urchins, make sure to record them separately and make a picture.
11. If in doubt if something is a *D. antillarum*: use the macro function of the camera to enlarge it, make a picture and send it to Alwin Hylkema (alwin.hylkema@hvhl.nl)
12. Put all *D. antillarum* in a petri dish
13. If all containers from 1 sample are examined:
    a. Make a picture of the sample ID on the analysis sheet.
    b. Put the petri dish on the sealed mm paper.
    c. Make a picture of all *D. antillarum* in the sample. If they are close together you can photograph them in groups. It is important that the photograph is straight from above, so move the *D. antillarum* away from the sides of the petri dish before you make the pictures. The outline of the *D. antillarum* and the mm paper should both be visible.
    d. Write down any unusual observation under "remarks" (e.g. parts of the sample missing etc)
14. Throw the other organisms and the seawater back in the ocean and rinse the containers so there are no (small) organisms left behind.
15. Repeat the procedure for the next sample.

Division into species for composition of patch

16. Fill 3 buckets with fresh sea water.
17. Divide the *Sargassum* into one of the buckets based on species/morphotype (Fig. 5).

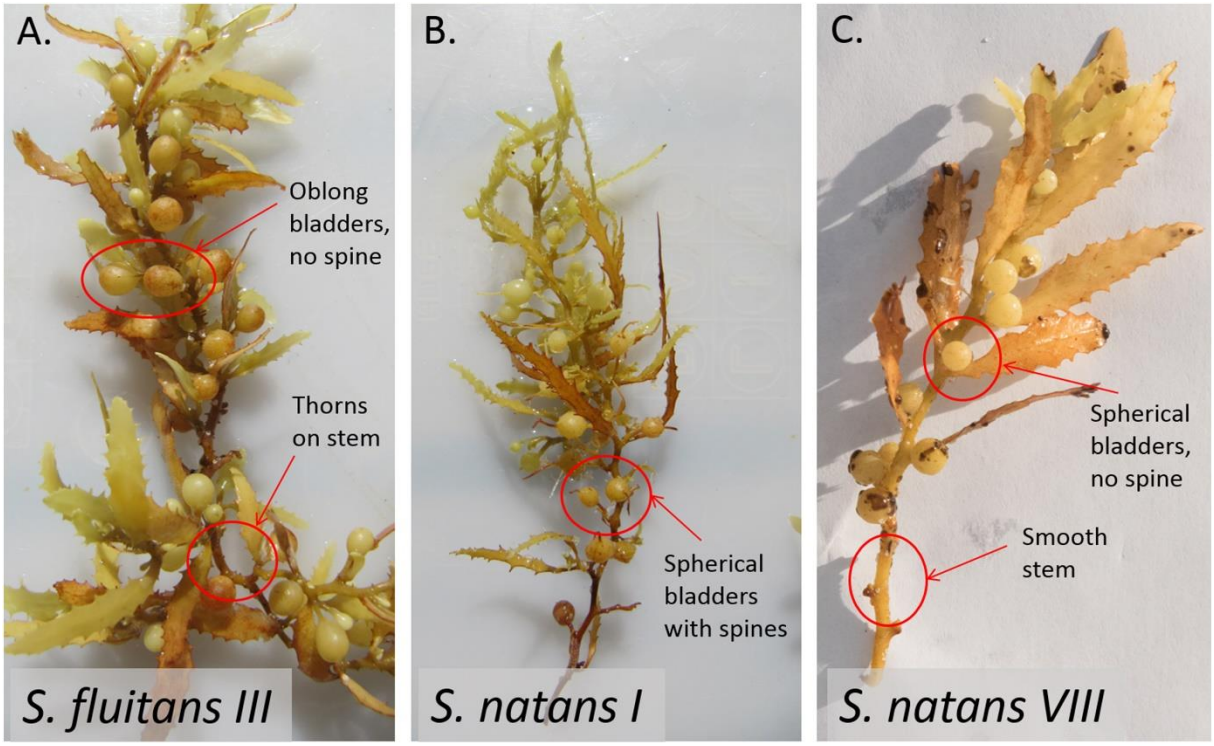


Figure 5: Characteristics and appearances of possible morphotypes in the samples (Govindarajan et al., 2019) Characteristics will be written on a whiteboard for extra clarity.

18. Per bucket, measure length of stalks and tally on the "*Sargassum* stalk length tally" sheet, everything under 3 cm belongs to primary stalk. Stalks longer than that count as individual side stalks and should be measured as such (Fig. 6). If ends of stalks have tiny sprouting buts, these count as part of the stalk and not as leaf.

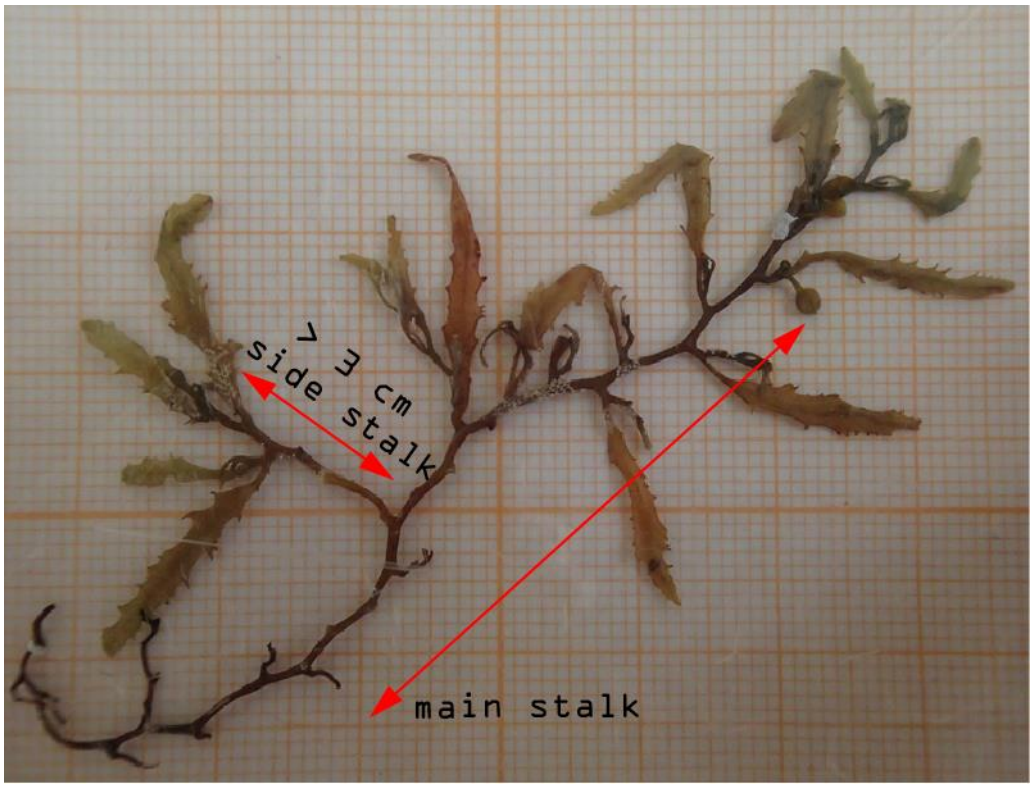


Figure 6: Division into main stalks and side stalks. Side stalks are identified as stalks longer than 3 cm and should be measured separately.

19. After all samples have been searched and all stalks have been measured, the *Sargassum* can be thrown away.

! Always insert your data on the same day!

Data entry:

1. Make a picture of the filled in analysis sheet (so we can always look back in the original data.
2. Open the data file.
3. Save the data file as a new version, include the date in the file name.
4. Enter all observations in the data file and save the data file.
5. Save the pictures:
    a. The picture of the analysis sheet should be stored in "photos and videos", "photos data sheets" and "period X, month year".
    b. The pictures of the *D. antillarum* should be saved in "Photos and videos", "size photos", "period X, month year", "date", "location".
    c. The pictures of unknown organisms should be saved in "Photos and videos", "size photos", "period X, month year", "unknown organisms". Make sure to add the sample ID (eg TR5) to the name of the photo.

References:

Govindarajan, A. F., Cooney, L., Whittaker, K., Bloch, D., Burdorf, R. M., Canning, S., ... & Siuda, A. N. (2019). The distribution and mitochondrial genotype of the hydroid Aglaophenia latecarinata is correlated with its pelagic *Sargassum* substrate type in the tropical and subtropical western Atlantic Ocean. *PeerJ*, *7*, e7814.

APPENDIX III

Protocol analysis *D. antillarum* settlement *Sargassum*

Adapted from "*Protocol analysis D. antillarum settlement collectors"* by Alwin Hylkema, May 2021

Materials:

1. Samples collected in 2 gallon zip-lock bags
2. 10 White tubs/ containers
3. Analysis sheet on waterproof paper + pencil
4. 3 buckets: one with clean, fresh sea water, one to throw away the analyzed samples and one extra
5. Pipette
6. Measuring cup
7. Sticks/skewers for sorting
8. Sealed mm paper
9. Camera
10. Small container (e.g. old yoghurt cup) to store *D. antillarum* after analysis

Methods:

After retrieving the *Sargassum* and bioball experimental units, all samples are analyzed **separately** and possibly within 4 hour after collection!

For bioballs:

1. Start with the samples that were collected the earliest.
2. Try to remove all organisms from the bioballs by shaking the sample thoroughly in the zip lock bag.
3. Pour the contents of the bag in a container. Make sure no organisms stick in the bag.
4. Put 1L of clean seawater in the next container and rinse the samples again.
5. Repeat steps 2-4 five times in total, use a different container after every rinse, so in the end you have 5 containers in use.
6. Place the containers on a table and let them rest for 1 minute
7. Search the containers carefully for organisms, identify them and record them on the analysis sheet. Use the document "Identification of marine organism in *D. antillarum* settlement traps".

For *Sargassum*:

1. Start with the samples that were collected the earliest.
2. Try to remove all organisms from the Sargassum by shaking the sample thoroughly in the zip lock bag.
3. Pour the contents of the bag in a container. Make sure no organisms stick in the bag.
4. Put 1L of clean seawater in the next container and rinse the samples again.
5. Divide the contents in small batches over several containers, as many as necessary to be able to search through the sample, and rinse the Sargassum thoroughly before throwing the Sargassum away.
6. Place the containers on a table and let them rest for 1 minute.
7. Search the containers carefully for organisms, identify them and record them on the analysis sheet, as with the bioballs.

The following organisms must be identified:

1. *D. antillarum*
2. Other sea urchins (up to species level if possible)
3. Crabs (Spider crab/crab other)
4. Hermit crabs
5. Shrimps (Mantis shrimp/shrimp other)
6. Lobster
7. Worms (fireworm/worm other)
8. Other crustacea
9. *D. antillarum* can be as small as 1 mm, so look really carefully! Also, if you find a *D. antillarum*, you can suck it up with the pipette, but be careful, because if you wait too long with putting it in the petri dish, the *D. antillarum* will adhere to the pipette.
9. Living *D. antillarum* adhere quickly to the container, if you shake the container carefully you can find them easily by looking at small round, red dots that are not moving. If you look more carefully, you can see the (striped) spines of the *D. antillarum*. Collect both living and dead *D. antillarum*.
10. We usually do not find other sea urchins, but you can always recognize *D. antillarum* because they have striped spines and are very red after settlement. They turn black within 2 weeks, so you can also find black *D. antillarum* (but these still have striped spines). If you find other sea urchins, make sure to record them separately and make a picture.
11. If in doubt if something is a *D. antillarum*: use the macro function of the camera to enlarge it, make a picture and send it to Alwin Hylkema (alwin.hylkema@hvhl.nl)
12. Put all *D. antillarum* in a petri dish
13. If all containers from 1 sample are examined:
    a. Make a picture of the sample ID on the analysis sheet (eg TR1).
    b. Put the petri dish on the sealed mm paper.
    c. Make a picture of all *D. antillarum* in the sample. If they are close together you can photograph them in groups. It is important that the photograph is straight from above, so move the *D. antillarum* away from the sides of the petri dish before you make the pictures. The outline of the *D. antillarum* and the mm paper should both be visible.
    d. Write down any unusual observation under "remarks" (e.g. parts of the sample missing etc)
14. Throw the other organisms and the seawater back in the ocean and rinse the containers so there are no (small) organisms left behind.
15. Repeat the procedure for the next sample.

Always insert your data on the same day!

Data entry:

1. Make a picture of the filled in analysis sheet (so we can always look back in the original data.
2. Open the data file.
3. Save the data file as a new version, include the date in the file name.
4. Enter all observations in the data file and save the data file.
5. Save the pictures:
    a. The picture of the analysis sheet should be stored in "photos and videos", "photos data sheets" and "period X, month year".
    b. The pictures of the *D. antillarum* should be saved in "Photos and videos", "size photos", "period X, month year", "date", "location".

c. The pictures of unknown organisms should be saved in “Photos and videos”, “size photos”, “period X, month year”, “unknown organisms”. Make sure to add the sample ID (eg TR5) to the name of the photo.

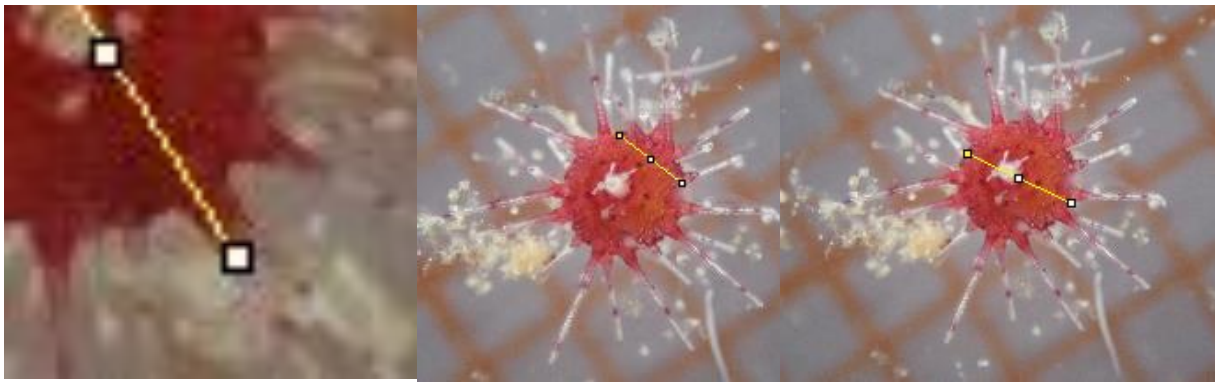
Samples after analysis:

1. Soak samples in fresh water for at least 24 hours.
2. Dry the samples.
3. Store the samples when dry.

***D. antillarum* measurement protocol ImageJ v1.0**
Alwin Hylkema, August 2019

This protocol describes the procedure to measure *D. antillarum* recruits with ImageJ. ImageJ is a freeware program, made to process and analyse images. ImageJ can be downloaded via this link: https://imagej.nih.gov/ij/download.html.

1. Open ImageJ and open the picture (File -> Open (CTRL + O)).
2. To set the desired variables go to: Analyze -> Set measurements…. Deselect all options and click on OK.
3. Select the line *Straight* (5th button from the left), and measure 10 mm on the mm paper next to the *D. antillarum*. To set the scale go to: Analyze -> Set scale…. Fill 'Known distance:' 10. Click on 'OK'.
4. To check if the scale is set right, measure again 10 mm on the mm paper. Press Analyze -> Measure (Ctrl + M) and check if the measurement is between 9.80 and 10.20. If not, set the scale again and repeat the control step.
5. Measure the diameter of the test (the body without the spines) of the *D. antillarum*. The measurement can be made in a random direction, but should go through the middle of the test. After you draw the measurement, press Analyze -> Measure (CTRL + M).



Wrong measurement, because the spines are included.

Wrong measurement, this line does not go through the middle

Correct measurement

6. Insert your measurements in the datafile "*D. antillarum* size data".