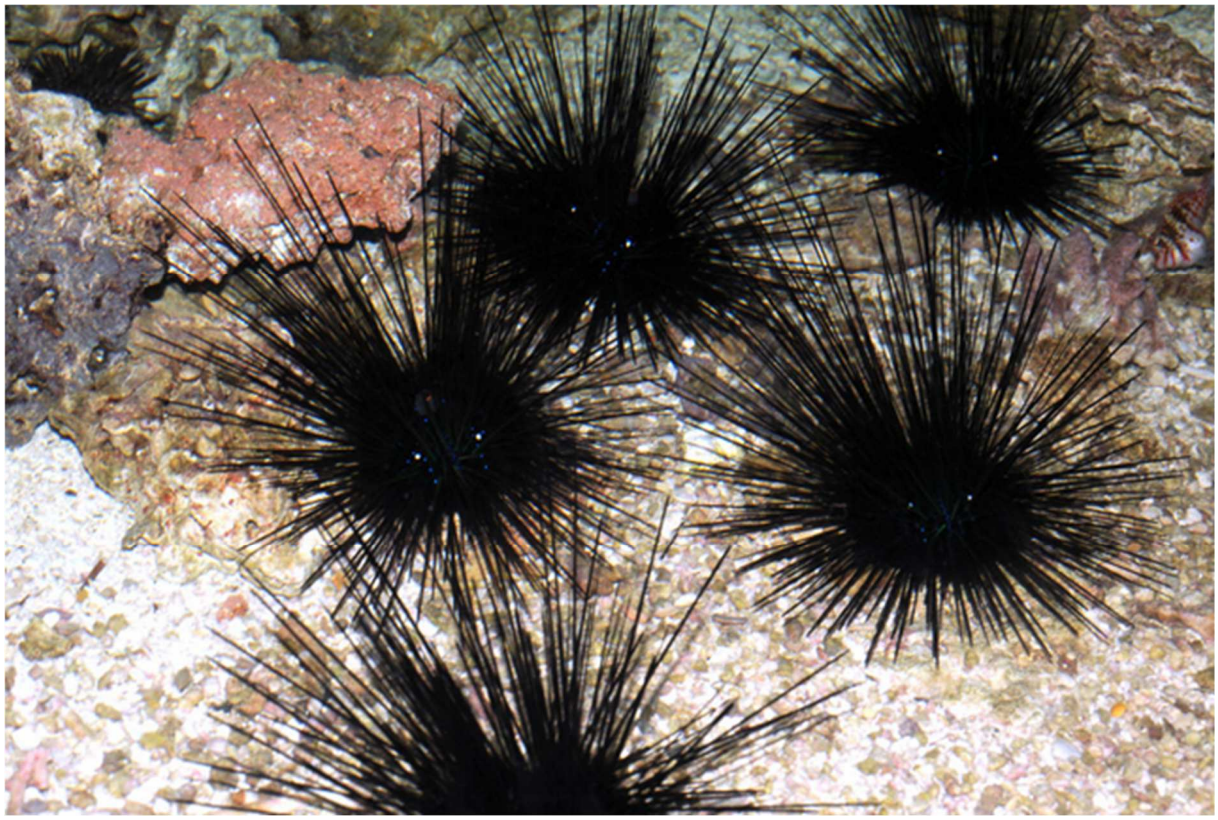
Research Report

Optimization of the culture of the long-spined sea urchin (*Diadema antillarum*) in order to maximize the larvae survival

The effect of different tank set ups on the survival rate of brine shrimp (*Artemia salina)* as a model organism for tropical sea urchin (*Diadema antillarum)* larvae



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26-08-2018

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Preface

We hereby present to you our final thesis report of the research for the optimization of the culture of the long-spined sea urchin (*D. antillarum*) in order to maximize the larvae survival, which has been performed and written on the University of Applied Sciences Van Hall Larenstein in Leeuwarden, The Netherlands.

During the process of performing this research, the help of a couple of persons has been crucial in order to come up with this result. We would like to thank all of these persons separately. First, we would like to thank Alwin Hylkema (Van Hall Larenstein) and Tom Wijers (Van Hall Larenstein) for their overall help, compassion and feedback they have given us during the process. Without their assistance, the report would have looked a lot different. Secondly, thanks goes out to Peter Hofman (Van Hall Larenstein) and David Goldsborough (Van Hall Larenstein) for their critical view on both the Biology and the Policy part of this research. A big thanks to Ans Schoorlemmer (Van Hall Larenstein) as well, for her support and helpfulness in thinking about possibilities for the facilities where the practical part of this research took place. Of course thanks goes out to Van Hall Larenstein as well for providing the opportunity of performing our final thesis.

As for external persons, firstly we would like to thank Jeffrey de Pauw (De Jong Marinelife), for the fast delivery of the Diadema specimens and Gerd Arnd for building and delivering the Kreisel tanks. Thanks to Martin Moe for all the documents regarding his research for optimizing larval survival of *D. antillarum* and his help and feedback during the process. At last, we would like to thank Paul Hoetjes (Marine biologist and Policy coordinator at Rijksdienst Nederland), for his help for the policy part of this research.

We hope you will enjoy reading this final thesis report.

Daniël Altemühl and Dion Vink

Summary

Coral reefs in the Caribbean have suffered massive losses of corals since the early 1980s due to a wide range of human impacts, such as explosive human populations growth, coastal pollution, global warming, overfishing, invasive species and diseases. The publication in the journal science in 2003 stated that live corals have been reduced from more than 50 % in the 70s to 10% today. One of the causes has been the enormous die off of *D. antillarum* (Long Spined Sea Urchin), an absolute keystone species on the reef, which keeps coral reefs healthy and clean from algae. So this research was set up, which has focused on the breeding techniques of the *D. antillarum* and the regulations regarding the restocking of cultured *D. antillarum* in the waters surrounding the islands Saba and St. Eustatius. Due to the poor available information about breeding these animals, research question were set up to determine which breeding setup would be the most suitable and what policies have to be taken into account when restocking. Three systems, kreisel tanks, plastic bottles and beakers on a shaking table were used to determine the system with the highest survival rate. Due to the fact that the spawning of the *D. antillarum* and *D. setosum* failed and no larvae were obtained, the choice was made to use *Artemia salina,* known as brine shrimp, as a model organism for the experimental part of this research. Immediately after the first results, the kreisel tanks turned out to be the most sufficient set-up. At the end of the experiment (13 days) the data and statistics have shown that the kreisel tanks were the most suitable, followed by the beakers on the shaking table. This did not mean that the plastic bottles had an insufficient result, as the amount of *Artemia salina* increased in each system, with the highest increase in the kreisel tanks, which are easier to maintain as well. As for the regulations concerning the restocking, the advice has been given that more research is needed in order to set up a detailed list of criteria that have to be met. At this moment, restocking is possible with the mandatory permits, but for the local permits, no criteria are set yet, as the restocking of this species has not been done before in the waters surrounding the islands Saba and St. Eustatius.

Inhoudsopgave

1. Introduction

Coral reefs in the Caribbean have suffered massive losses of corals since the early 1980s due to a wide range of human impacts, such as explosive human population growth, coastal pollution, global warming, overfishing, invasive species and diseases. The consequences are widespread collapses of coral populations, increases in seaweeds, outbreaks of coral bleaching and diseases and failure of corals to recover from natural disturbances such as hurricanes. The publication in the journal Science in 2003 that live corals had been reduced from more than 50 % in the 1970s to just 10 % today, sett of the alarm bells, but this had been going on for a much longer time (Jackson, et al., 2012). One of the causes of this massive loss has been the great die off of *Diadema Antillarum* (Long spined Sea Urchin) (Figure 1) in 1983, during which almost 97% of the at that moment existing *D. antillarum* in the Caribbean died (Puckett, 2002).

Figure 1 *Diadema antillarum* (St. John Snorkeling, 2017)

D. antillarum has an important role in the Caribbean and is a so called keystone species. It mainly grazes on fast growing algae on the reefs and without the presence of *D. antillarum* the corals die due to being overgrown with algae. It is often thought that there is no other single species on the coral reefs that has such great effects on other organisms in the same environment (Moe, In Prep. 2017(2); Lessios, 1988). The die off of *D. antillarum* caused an explosive growth of algae, causing large parts of the reef ecosystems to deteriorate (Puckett, 2002). There is some strong circumstantial evidence that a waterborne, host specific pathogen was responsible, but the connection between these pathogens and the epidemic was not considered conclusive (Lessios, 1988). At this moment, the population size is only 12 % of in 1983 (Lessios, 2015), so there is a recovery of the population, but it is very slow.

Culturing and restocking *D. antillarum* might be key to restoring the populations, but currently, there is not a lot of information available about the breeding of sea urchins in captivity. In nature, *D. antillarum* reproduce from early summer till early winter, with the peak in mid-summer. *D. antillarum* almost always spawn at the new moon, probably because of an increase of water temperature (Bauer, 1976; Farland, 2018; Garrido, et al., 1999; Iliffe & Pearse, 1981; Reefbuilders, 2015). Martin Moe (hobbyist and aqua culturist) has researched breeding possibilities of sea urchins for multiple years. Especially *D. antillarum* had his interest and therefore he is writing a breeding manual which was of great use. It has been shown that *D. antillarum* spawn at a sudden increase of temperature (+/- 5 degrees Celsius) regardless of the moon cycle. This is also sporadically noticed by hobbyists and wholesalers. At this moment, the change of temperature seems to be the key to control the spawning of *D. antillarum* (Moe , In Prep. 2017; Moe, In Prep. 2017(2); Reefbuilders, 2015). But for the grow out process there are a few things that are causing problems. The problem at the moment is to produce juvenile/mature animals from larvae who are really sensitive (Moe, In Prep. 2017; Moe, In Prep. 2017(2)). Also the sensitivity to metals and poor water quality can be disastrous to larvae survival and should be take into account when trying to culture *D. antillarum* larvae (Bielmyer, et al., 2005; Moe, In Prep. 2017).

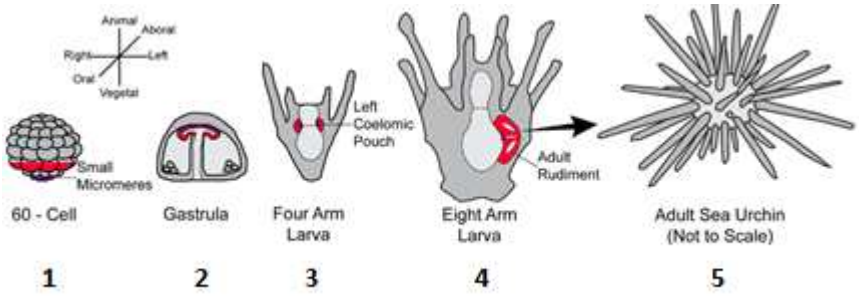


Figure 2 The life phases of the *D. antillarum* (Kiddle, 2018)

Most sea urchins, including *D. antillarum* have a pelagic life phase in which they are transported by the currents and thereby have access to micro-algae as their food (Figure 2, Stage 1 - 4). The larvae will stay close to the surface and can travel great distances being transported by the currents. This stage is very hard to control when trying to reproduce in captivity, because the larvae need a constant current and a constant abundancy of food (algae). When this stage is passed (Stage 5), juveniles will sink to the bottom and try to find a suitable place to settle and eventually grow out to an adult. Due to the until now almost uncontrollable egg and larval stage, this juvenile stage is, in captivity, only seen on a very small scale (Bauer, 1976; Moe, In Prep. 2017; Puckett, 2002).

In culturing different kind of sea urchins, but also for *D. antillarum, Rhodomonas sp.* turned out to be a good food source and a constant amount of food availability was crucial for larval survival (Cameron & Schroeter, 1980; Moe, 2017; Salas-Garza, et al., 2005; Vaughan, 2010; Wolcott & Messing, 2005). For multiple pelagic species, kreisel tanks are used for breeding the larvae (de Montgolfier, et al., 2005; Goldstein & Nelson, 2011; Harvey & Morrier, 2013; Preininger, et al., 2014). Kreisel tanks are circular tanks without any corners, preventing delicate creatures from injuries. Besides that, kreisel tanks make great homes to pelagic creatures that need a constant flow in order to survive, which is the case for the *D. antillarum* larvae (Fishlarvae.org, 2017). Another set up that is used for culturing brine shrimp (*Artemia salina*) is the brine shrimp tank, which can be built out of plastic bottles (saltwateraquariumblog.com, 2009; solidgoldfish.com, 2013). Brine shrimp are comparable to *D. antillarum* due to their pelagic life phase and size, their size is comparable to the size of the *D. antillarum* larvae at an age of 25 to 35 days. A third set up that is used for culturing a different species of sea urchin, *P. miliaris,* is the shaking table. Beakers are placed on the shaking table which maintains a constant circular movement of the beakers (Anselmo, 2012).

Currently, it is not clear what policies have to be taken into account when restocking wild populations of *D. antillarum* if culturing is a success. The focus of this research has been on the restocking policies of the Dutch Caribbean Islands Saba and St. Eustatius (Statia). These islands are the main focus of the AROSSTA (Artificial reefs on Saba and Statia) project, for which this research has been done. The AROSSTA project focusses on determining how artificial reefs can contribute to the recovery of coral reef ecosystems on Statia and the Saba bank (Hylkema, 2018). But because until shortly, nobody has ever cultured *D. antillarum* with the purpose of restocking wild populations, no desktop study had been done for the known information about these policies. It is crucial to create insight in the known policies and bottlenecks in order to make it clear for future research what to take into account when culturing *D. antillarum* with the purpose of restocking.

2. Problem Description

In order to restore Caribbean reef ecosystems, it might be necessary to restock the ecosystem with cultured *D. antillarum*. However, when *D. antillarum* is being bred, larval survival is very low and very few of the fertilized eggs successfully settle as juvenile sea-urchin.

Currently, the few researches that have been done to optimize the survival rate of *D. antillarum* larvae, have all used kreisel tanks as a hatchery for the larvae (Capo, et al., 2009; Moe M. , 2017; Moe M. A., In Prep. 2017; Moe M. A., In Prep. 2017(2). But for the other set ups, the brine shrimp larvae tank and the shaking table, so far, no research has been done to find out whether these set ups might have a positive effect on the larval survival of *D. antillarum*.

Another uncertainty is the current available information and possible bottlenecks for policies regarding the restocking of cultured *D. antillarum* on the Dutch Caribbean Islands Saba and St. Eustatius on the reefs where *D. antillarum* population is very low or even vanished (Puckett, 2002).

Due to the fact that the spawning of the *D. antillarum* and *D. setosum* failed and no larvae were obtained, the choice was made to use *Artemia salina,* known as brine shrimp, as a model organism (Figure 3). Brine shrimp are comparable to *D. antillarum* due to their pelagic life phase and size, their size is comparable to the size of the *D. antillarum* and *D. setosum* larvae at an age of 25 to 35 days, during which most problems occurred (Artemia international LLC, 2018; Moe, In Prep. 2017). Besides that, they share the high sensitivity for metals and pH (Bielmyer, et al., 2005; Hirota & Gajbhiye, 1990; Macrae & Pandey, 1991).

Figure 3 *Artemia salina* (Wikipedia, 2018)

3. Aim

The first goal of this research is to provide insight in which of the larvae tanks has the highest larval survival rate, in order to optimize the culture of brine shrimp (*Artemia salina)* as a model organism for *D. antillarum* larvae to eventually research the possibilities of restocking wild populations.

The second goal is to provide insight in the policies and bottlenecks that have to be taken into account when restocking wild populations with cultured *D. antillarum* on the Dutch Caribbean Islands Saba and St. Eustatius. This information will be converted to an advise for nature management organizations.

4. Research Questions

For this research, two research questions have been formulated:

1. **Which of the three systems, kreisel tanks, plastic bottles and the beakers on the shaking table, has the highest survival rate when culturing *Artemia salina* as a model organism for *D. antillarum* larvae?**
2. **Is it possible to restock wild populations with cultured *D. antillarum* on the reefs surrounding the Dutch Caribbean Islands Saba and St. Eustatius while complying to the current policies?**
    - **What information is already known about the policies regarding restocking of wild populations with cultured species?**
    - **What are the bottlenecks regarding the policies about restocking wild populations with cultured *D. antillarum* on the Dutch Caribbean Islands Saba and St. Eustatius and how to minimize the bottlenecks?**

5. Methods

This part of the report has been divided into different sections, all describing a different part of the culturing process. All information that has been gathered during the process has been noted and written down and eventually converted into digital information in spreadsheets.

5.1 Experimental set-up

A total of three larvae tank set ups have been used for this research. The first set up were the kreisel tanks (Figure 4).The plan was to build the kreisel tanks for financial reasons, so different material was purchased. Unfortunately, the material that was used turned out to be insufficient, as it could not handle the water pressure in the tank. Due to the fact that a kreisel tank set up turned out to be very sufficient for culturing larvae, a desktop study was set up in order to find a kreisel tank producer. This eventually led to Gerd Arnd, owner of aquarienBastelei and a hobbyist that builds kreisel tanks for aquaculture purposes (Arnd, 2018). The tank has to be placed in an aquarium. For this research, both kreisel tanks were placed in one large aquarium with a volume of 60 L (Figure 6). For these kreisel tanks, an air pump is used to circulate the water, and a 63 µm mesh on the side enables water exchange, but keeps the *Artemia salina* in the tank. Water parameters were checked daily and a 10% water change was done when the parameters were not optimal. Water change was done with a small hose and taken out of the large tank, in between the kreisel tanks (Figure 9). This was done to prevent *Artemia salina* from being taken out of the set up. Artificial sea water with the right parameters was then used to fill up the bottles. In case of high salinity, osmosis water was used to fill up the bottles and take down the salinity levels.

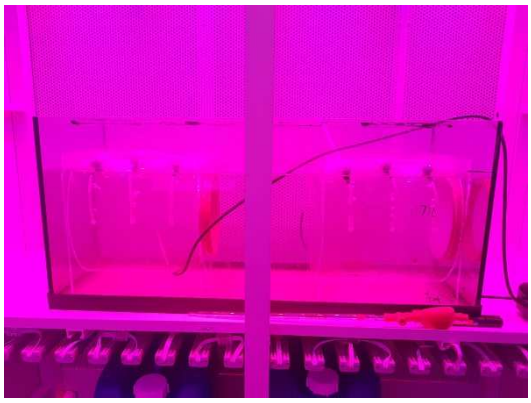


Figure 4 The kreisel tank set up

Figure 5 The plastic bottle set up

The second set up that has been used were the plastic bottles (Figure 5). Simple soda bottles, purchased at the local grocery shop, were emptied, cleaned and the top with the screw cap was removed. A large air pump, with a divider was used to keep the oxygen level in each bottle optimal. Small hoses with a simple straw at the end were inserted into the bottles, blowing small bubbles up, which made the water circulate in the bottles. Water parameters were checked daily and a 10% water change was done when the parameters were not optimal. Water change was done with a small hose from the top layer of the water, after the air pump was shut off so the *Artemia salina* would sink a little bit. This was done to prevent *Artemia salina* from being taken out of the set up (Figure 9). Artificial sea water with the right parameters was then used to fill up the bottles. In case of high salinity, osmosis water was used to fill up the bottles and take down the salinity levels.

Figure 6 The shaking table set up

The third and last set up that was used was the shaking table (Figure 6). Four beakers of each 500 ml of water containing *Artemia salina* were placed on a shaking table

Figure 7 Water change for all set ups

which was then set at a speed of 150 rotations per minute. No air pump was used in this set up, due to the effect that the shaking of the beakers should be sufficient enough to maintain oxygen levels in the water. It also provides a circulation in the water column. Water parameters were checked daily and a 10% water change was done when the parameters were not optimal. Water change was done with a small hose from the top layer of the water, after the shaking table was shut off so the *Artemia salina* would sink a little bit (Figure 7). This was done to prevent *Artemia salina* from being taken out of the set up. Artificial sea water with the right parameters was then used to fill up the bottles. In case of high salinity, osmosis water was used to fill up the bottles and take down the salinity levels.

5.2 Hatching techniques

Figure 8 The *Artemia salina* culture set up

In order to obtain *Artemia salina*, *Artemia salina* eggs were purchased via Voervoorvis (Bol.com, 2018). Once these eggs arrived, the hatching process started. Two plastic bottles were filled with artificial seawater with a salinity of 31 ppt (parts per thousand), a pH of 8 and were placed in a climate room with a temperature of 25 degrees Celsius (Brine shrimp direct, 2018). Before adding the *Artemia salina* eggs, the water quality was measured. Both bottles were equipped with an air pump to maintain the oxygen levels in the water column. To each bottle, a small teaspoon of *Artemia salina* eggs (approximately 1 gram) was added (Figure 8). After 24 hours, the air pump was shut off, so the *Artemia salina* would sink to the bottom and the unhatched eggs would float on top of the water column. The unhatched eggs were removed as much as possible and the air pump was turned on again, so the *Artemia salina* would be divided equally in the water column. 10 one ml samples were taken randomly and observed under a binocular to determine the amount of *Artemia salina* per ml (Figure 9). To simplify the dividing of the *Artemia salina*, only the plastic bottle containing the most *Artemia salina* per ml was used. For the different tanks, the amount of water containing *Artemia salina* that had to be added in order to have a *Artemia salina* density in each tank of 0,1 larvae/ml, which turned out to be the best larval density for *D. antillarum* (Moe, In Prep. 2017), was determined. Each kreisel tank has a volume of 13,74 L (Arnd, 2018), each plastic bottle a volume of 1 L and each beaker on the shaking table a volume of 0,5 L. A density of 0,1 larvae per ml means a total amount of 1374 *Artemia salina* in the kreisel tanks, 100 *Artemia salina* in the plastic bottles and 50 *Artemia salina* in the beakers on the shaking table. In the samples that were taken from the *Artemia salina* run, an average of 19 *Artemia salina* per ml was found in 10 samples. To determine the amount of *Artemia salina* containing water that had to be added to the different set ups, the total amount of *Artemia salina* per set up was divided by the average amount in the *Artemia salina* run, using the formula: A / B = C, with A being the desired amount of *Artemia salina* in the set-up, B being the average amount of *Artemia salina* found in the samples of the

Figure 9 Determining the amount of *Artemia salina* larvae per ml

hatching bottles and C being the amount of *Artemia salina* containing water from the *Artemia salina* run that had to be added.

Kreisel tanks
1374 (A) / 19 (B) = 72,32 ml (C)

Plastic bottles
100 (A) / 19 (B) = 5,26 ml (C)

Beakers on the shaking table
50 (A) / 19 (B) = 2,63 ml (C)

5.3 Feeding process

As said in the introduction, in culturing different kind of sea urchins, but also for *D. antillarum, Rhodomonas sp.* turned out to be a good food source and a constant amount of food availability was crucial for larval survival (Cameron & Schroeter, 1980; Moe, 2017; Salas-Garza, et al., 2005; Vaughan, 2010; Wolcott & Messing, 2005). Besides the use of *Rhodomonas sp.* for sea urchin larvae, *Rhodomonas sp.* can also be used when culturing *Artemia salina* (Seixas, et al., 2009). *Rhodomonas sp.* was provided by Stichting Zeeschelp in 1 L bottles (Stichting Zeeschelp, 2018). The bottles with algae were placed in the same climate room as the set ups and equipped with an air pump to keep the oxigen levels in the bottles optimal.

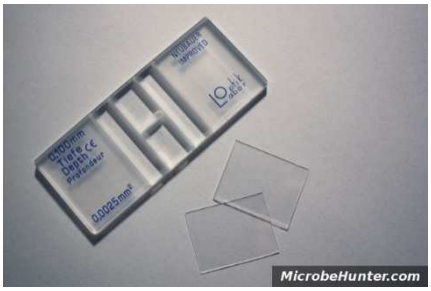


Figure 10 A Hemocytometer (burker turk) (Abcam, 2018; Cell Culture Chronicles, 2016)

Figure 11 Determining the amount of algae per ml

To determine the density of the algae in the bottles, a Hemocytometer (burker turk) was used (Abcam, 2018; Cell Culture Chronicles, 2016)(Figure 10). A 1ml sample, taken with a pipette, was added on the Hemocytometer and examined under a photomicroscope (Figure 11). After this, the amount of algae water that had to be added to the different set ups to achieve a concentration of 30.000 cells/ml was calculated and that amount was added to the different set ups (Brand.de, 2018). The algae concentration in the different set ups was measured daily, to maintain the concentration of 30.000 cells/ml. In case the concentration of algae in the different set ups was less than 30.000 cells/ml, the missing quantity was added by using a pipette.

5.4 Larval survival

To determine larval survival, sampling of the *Artemia salina* was done with a pipette in 10 ml samples. These samples were put in petri dishes and counted. The *Artemia salina* were large enough to be counted with the eye, so no binoculars or microscopes were used for the counting process. A total of five samples were taken on different locations in the set-ups (Figure 12) on a daily basis. After 13 days, the last counting took place. This final counting was used as the end result for the statistical analysis of this research. Counting was done on 9 out of 13 days, due to the fact that during the weekends sampling and counting was not possible. This information can be seen in Appendix I.

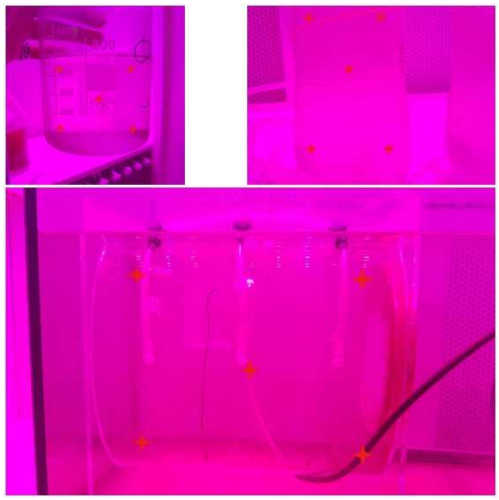


Figure 12 Sampling locations in the different set-ups

5.5 Data analysis

For this research, IBM SPSS statistics 25 was used. The data gathered during this research was the amount of *Artemia salina* alive. With the amount of Artemia between the first and last day the survival rate can be calculated (Amount of *Artemia salina* on the last day divided by the amount of *Artemia salina* on the first day) and tested for significance. p values < 0.05 were considered statistically significant. (Laerd Statistics, 2013; SPSS Handboek, 2017).

For this research, the different set ups, kreisel tanks, plastic bottles and beakers on the shaking table, were used as independent variables. The survival rate was used as dependent variable. To determine the difference of the dependent variable for each of the three independent variables, the One-way ANOVA test has been used (Medcalc, 2018; SPSS Handboek, 2017).

First, a test for equality of variances (Levene's test for equality of variances) was used. The outcome of this test was that the data was not significantly spread (<= 0,5), which means that the data is normally distributed Table 1). This also means that the use of the One-way ANOVA test is accepted (SPSS Handboek, 2017).

Levene's test	df1	df2	Sig.
1,050	2	7	0,399

Table 1 Levene's test for Homogeneity of variances

If the ANOVA returns statistically significant results (p= <0.05), the alternative hypothesis (H1) will be accepted, which means that there are at least two group means that are statistically significantly different from each other (Laerd Statistics, 2013; SPSS Handboek, 2017). In that case, the bonferroni post-hoc tests will be performed for pairwise comparisons.

5.6 Restocking policies

In order to provide insight in which policies have to be taken into account when restocking wild populations with cultured *D. antillarum,* a literature and desktop study has been performed. With the help of this information, the bottlenecks were identified and described.

First of all, all the known information and policies regarding the restocking of *D. antillarum* and marine species in general were listed. This information was selected on a number of criteria:

- is about the Caribbean.
- is about restocking, reintroduction, translocation and restauration of marine species, if possible for *Diadema* in general.
- is about current policies, frameworks, guidelines and or regulations.
- is scientific information, or at least written by a governmental or conservational organization or institute.

Multiple keywords were used and combined to find all the known and available information about the policies concerning this topic using Google and Google Scholar (Table 2).

Agency	Aquaculture	Bodies
Conservation	Culturing	*Diadema* (*antillarum/setosum*)
Dutch Caribbean (Islands)	EU	Exclusive economic zone (EEZ)
Frameworks	Governmental	Guidelines
(Il)legal	Legislation	Local
Management	Marine protected area (MPA)	Marine species
Municipalities	National	Permits
Policies	Reefs (Coral)	Regional
Regulation(s)	Reintroduction	Restauration
Restocking	Saba	Sea Urchin(s)
Statia / St. Eustatius	Stocking	Translocations

Table 2 Keywords used to find information

All this information was then selected on relevance for the *D. antillarum* study. Secondly, possible bottlenecks were determined. At last, all the information was then converted into a detailed advise for nature management organizations by setting up a list of criteria and policies that have to be taken into account in order to overcome the bottlenecks, which will also answer the second research question.

6. Results

All the output that was provided by the SPSS software can be found in Appendix II.

6.1 Results *Artemia salina* experiment

In this part of the results section the results of the One-way ANOVA are shown and explained. These results will eventually answer the first research question. This answer can be found in the conclusion.

In all set-ups, the *Artemia salina* density increased during the experiment. This can be due to reproduction of the *Artemia salina* and the hatching of accidently added eggs. The highest increase in density of *Artemia salina* was found in the Kreisel tanks, where the density almost multiplied by five from 0,1 to 0,445 *Artemia salina* per ml in 13 days. This was also measurable by the amount of algae that had to be added each day, which was much more per ml per kreisel tank than per ml per beaker or plastic bottle. In the first table (Table 3) of this section, the starting amount of *Artemia salina* of each tank of each set up can be seen, as well as the amount of *Artemia salina* after 13 days.

System setup	Starting amount *Artemia salina*	Amount *Artemia salina* after 13 days
Kreisel1	1375	6320
Kreisel2	1375	5908
Plastic bottle	100	230
Plastic bottle	100	240
Plastic bottle	100	250
Plastic bottle	100	210
Beaker	50	145
Beaker	50	140
Beaker	50	155
Beaker	50	160

Table 3 The starting amount of *Artemia salina* and the amount after 13 days

This data was then used to perform the One-way ANOVA test, which resulted in a significance of p<= 0,05 (Table 4). This means that there is a significant difference between the survival rates of the different set ups, F(2,7) = 105.646, p = ,000. The test shows that at least one system differs from the rest. A Post Hoc Test can determine which set-ups differ from others.

Surival rate	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,059	2	,029	105,646	,000
Within Groups	,002	7	,00		
Total	,061	9			

Table 4 One way Anova Grouping Results

To determine the mutual differences between the different variables, the Bonferroni Post Hoc Test was used. This test showed that the difference between the beakers and the plastic bottles is less significant (p = ,003) compared to the comparison of the other set ups (All p = ,000)(Table 5).

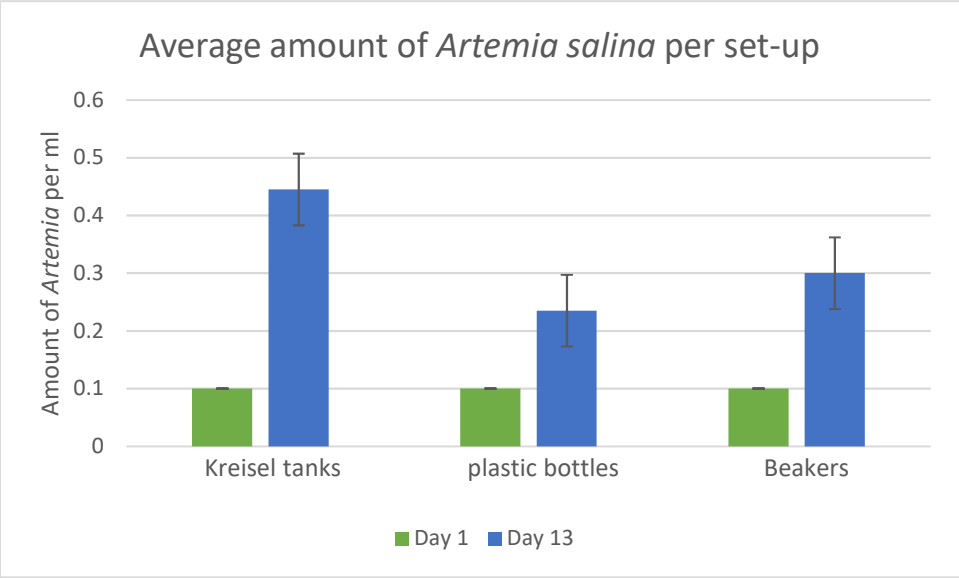
Factor	Against factor	Mean difference (Factor – Against factor)	Std. Error	Sig.	95% confidence interval, lower bound	95% confidence interval, upper bound
Kreiseltank	Plastic bottle	,21000-	,01445	,000	,1648	,2552
Kreiseltank	Beakers	,14500-	,01445	,000	,0998	,1902
Plastic bottle	Kreiseltank	-,21000-	,01445	,000	-,2552	-,1648
Plastic bottle	Beakers	-,06500	,01180	,003	-,1019	-,0281
Beakers	Kreiseltank	-,14500-	,01445	,000	-,1902	-,0998
Beakers	Plastic bottle	,06500-	,01180	,003	,0281	,1019

Table 5 The mutual differences between the different set ups

The Kreisel tanks had by far the highest mean of *Artemia salina* per ml after 13 days (0.445 *Artemia salina*/ml) followed by the beakers on the shaking table (0.3 *Artemia salina*/ml) followed by the plastic bottles (0.235 *Artemia salina*/ml)(Table 6)(Graph 1).

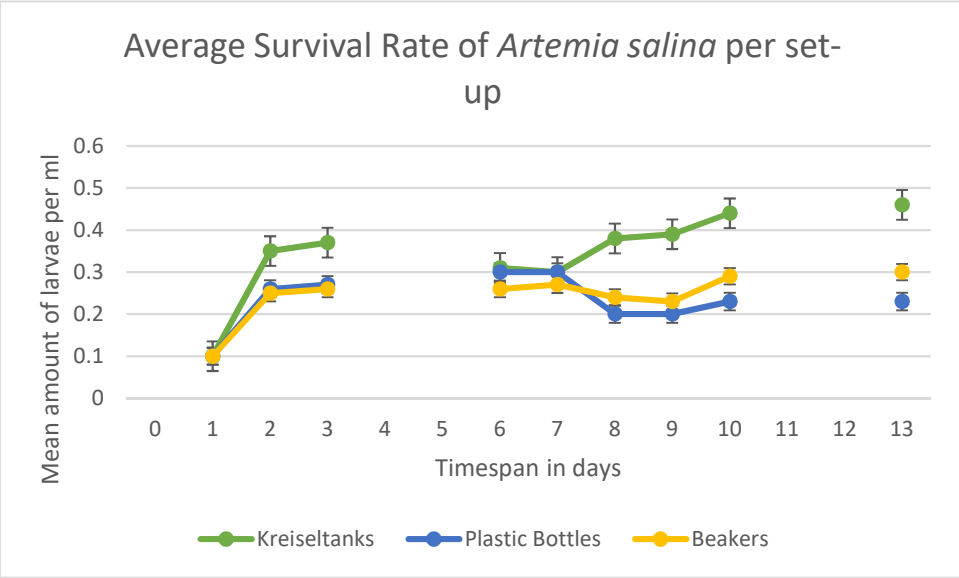
System	Starting percentage	Amount per 1 ml on first day	Growth percentage	Amount per 1 ml on last day
Kreiseltank	100%	0,1	445%	,445
Plastic Bottle	100%	0,1	233%	,235
Beaker	100%	0,1	300%	,3

Table 6 Survival rate of *Artemia salina*



Graph 1 Mean *Artemia salina* per 10 ml after 13 days

In the end, these results suggest that different set ups have different effects on the survival rate of *Artemia salina. Artemia salina* shows a positive growth rate, which can be seen in Graph 2. As can be seen in the graph above (Graph 2), on day 4, 5, 11 and 12, no data is shown. This was due to the weekends, in which measuring was not possible. Besides measuring the amount of *Artemia salina*, checking the water quality and algae concentration was not possible to. Due to this, salinity levels rose during these days, but that did not seem to have any great effects on the *Artemia salina*.



Graph 2 Average survival rate of *Artemia salina* per set-up

6.2 Results Restocking policies

6.2.1 Known Information

For this part of the results, first, the known information is listed. This was done in order to determine possible bottlenecks, which is the second part of this chapter. At last, possible solutions for these bottlenecks will be stated. To understand the possible bottlenecks, it is important to understand the definition of restocking animals into the wild. Restocking is a term used for animals that are bred or wild caught with the purpose of releasing them in the desired area, for example for conservation purposes (Richard, sd).

Information regarding restocking

An example of a species in the Caribbean that has been restocked/translocated is the Lesser Antillean Iguana, which was almost extinct on some of the Caribbean islands. At the moment nature organisations in collaboration with government bodies are restocking/re-introduction the Iguana's on some Islands with the help of the IUCN guidelines. These iguana's were bred in captivity on other islands (Vique, 2018; The Anguillian, 2016).

Convention on international trade in endangered species (CITES)

When culturing on a location in a different country or continent, research has to be done to find out whether the species is on the CITES (Convention on International Trade in Endangered Species) list. This international list determines which species are not to be traded or transported. If the species is on this list, a special CITES permit is necessary. This has been the case for the Lesser Antillean Iguana (IUCN, 2017). This made it illegal to just transport the iguana from one island to another. A permit was needed before the transportation was possible; therefore the collaboration between different stakeholders was needed to find the solutions together. The International Union for Conservation of Nature (IUCN) has set up guidelines which can be used when restocking, reintroducing. (IUCN, 2013). These guidelines were partly used in the Lesser Antillean Iguana Project. A lot of research was done to cover all possible problems such as genetics, population differences and more.

Specially Protected Areas and Wildlife (SPAW)

This protocol is a part of the Carthagena convention, a treaty for the protection and development of the marine environment in the Caribbean. The convention and its protocols constitute a legal commitment by the participating governments to protect, develop and manage their common waters individually or jointly (Caribbean Environment Programme, 2015; Van Gils & Schoenmaeckers, 2010). In case of the *D. antillarum*, trading and transporting is legal, as long as the right transportation and exportation permits are obtained.

IUCN Guidelines

There is a chance, that just as for the Lesser Antillean Iguana, the habitat management bodies will use guidelines set up by the IUCN for restocking the *D. antillarum*. These guidelines are not specifically set up for marine species, but for all species.

The following guideline sections are usable and probably also used by the marine parks on the restocking of the *D. antillarum* (IUCN, 2013).

- **IUCN guideline Section 3: Deciding when translocation is an acceptable option.**

This section is about clarifying the positive and negative effects of restocking the *D. antillarum*. The ecological, social and economic risks the restocking could cause have to be determined. Besides that, it has to be proven the previous threat is identified and sufficiently reduced or removed. The absolute risk must be balanced against the scale of expected benefits.

- **IUCN guideline Section 4: Planning and translocation**

Clear defined goals should be made. Conservation translocation should follow a logical process from initial concept to design, feasibility and risk assessment, decision-making, implementation, monitoring, adjustment and evaluation.

- **IUCN guideline section 5: Feasibility and design**

The primary focus of translocation planning will be the desired performance of the species in terms of either its population performance, behavior and / or its ecological roles after translocation. However, the design of the proposed translocation will be subject to both opportunities and constraints and all will influence the feasibility of the proposed operation. Feasibility assessment should cover the full range of relevant biological and non-biological factors.

- **IUCN guideline section 6: highly detailed risk assessment**

- **IUCN guideline section 7: Release and implementation**

A translocation should include a highly suitable area. Implementation should therefore take into account the aspects covered in guideline section 4,5,6 and 8 and particularly those that include legal requirements, public engagement, habitat management(marine parks), sourcing and releasing organisms, interventions and post-release monitoring.

- **IUCN guideline section 8 : monitoring and continuing management**

Genetic, health and mortality monitoring should be done regularly to identify progress. Also social, cultural and economic monitoring to assess attitude towards the translocation should be done.

6.2.2 Possible Bottlenecks

In order to restock wild populations, in this case of *D. antillarum,* with cultured individuals, a few things have to be taken in to account.

- First the transporting or trade of an endangered species can only be done if a permit is required, which contains the release of the CITES. CITES consists out of a set of appendixes which all have their own requirements. At this moment *D. antillarum* is not listed in the CITES at all, so no permit is needed. But with the rapid coral reef declining there is a possibility of the keystone species *D. antillarum* being listed on the CITES soon. It could for example be listed under appendix ll. Appendix ll is the list of species that are not necessarily threatened with extinction or rapid decline at the moment, but that might change unless trade is closely controlled. Also appendix lll is a possibility when listing *D. antillarum*. Appendix lll lists species that are at the request of parties regulated in the trade, that needs the cooperation of other countries to prevent unsustainable or illegal exploitation. In the worst case the species will be listed under appendix l, which means that the species is one of the most endangered among all CITES-listed. They are threatened with extinction and CITES prohibits any trade in specimens of these species (CITES, 2018).

- Without the right transportation and exportation permits, trading and transporting of *D. antillarum* is illegal according to the SPAW protocol, which is signed by the Kingdom of the Netherlands, which also included Saba and St. Eustatius (2.Caribbean Environment Programme, 2015).

- Simply restocking species in nature is not legal by the Wildlife act of 1975. In this act it is stated that to translocate a species permit is needed (VIC State Government of Victoria, 2018).

- To research and monitor the project a research permit is needed. This is needed before any organism can be taken out of the sea for research. Also if scuba diving is needed to monitor, a scientific research permit is again needed (VIC State Government of Victoria, 2018).

6.2.3 Possible solutions for these bottlenecks

- At this moment CITES is not a problem for restocking *D. antillarum*. But if in the soon future it will become listed in one of the CITES appendixes a permit request should be done. CITES provides permits for exceptional cases. For example the scientific research which is needed. In these exceptional cases, trade may take place if it is authorized by the granting of both an import permit and an export permit (re-export certificate). In article VII (article about exceptional cases criteria) of CITES it does not mention any exception about restocking a species for conservative needs (CITES, 2018). If the *D. antillarum* will be listed in one of the CITES appendixes, Paul Hoetjes, Marine biologist and Policy coordinator at Rijksdienst Nederland, can help out with the exceptional cases of CITES. All permits can be requested via Paul Hoetjes. This is further specified in the law principles nature conservation and protection BES (Overheid.nl, 2014).

- Without the right transportation and exportation permits, trading and transporting of *D. antillarum* is illegal. To obtain this permit, first of all the species should not be CITES listed (or an exceptional CITES permit is needed) and secondly a permit to trade and transport *D. antillarum* on the islands themselves is requested at the local marine parks. For Saba Kai Wulf, Marine park manager at the Saba Conservation Foundation (SCF)(SCF, 2018), is the person to contact for this permit. For St Eustatius this is Jessica Berkel, Marine park manager at STENAPA (STENAPA, 2018).

- When the moment of restocking is near, a permit for restocking the cultured *D. antillarum* should be requested at the local marine parks of both islands. Under section 28A of the wildlife act it is mentioned that the secretary of DEPI (Department of Environment and Primary Industries) may authorize the collection, keeping and breeding of native wildlife, taking of samples from and marking and experimentation on native wildlife, provided that the authorization is necessary for, among other things conservation, protection research and management. The marine parks claim that they are the authorizing party in case of the restocking on both islands (Duncan, 2013).

- A permit for doing research in the focus area has to be requested at Rijksdienst (BES) (Overheid.nl, 2014). To locally monitor the focus area while scuba diving a permit has to be requested at the local marine parks mentioned before. As can be seen in the figures above there are some marine protected areas in the coastal waters of both islands.

On the next page, a flow diagram (Figure 13) shows all permits that have to be requested.

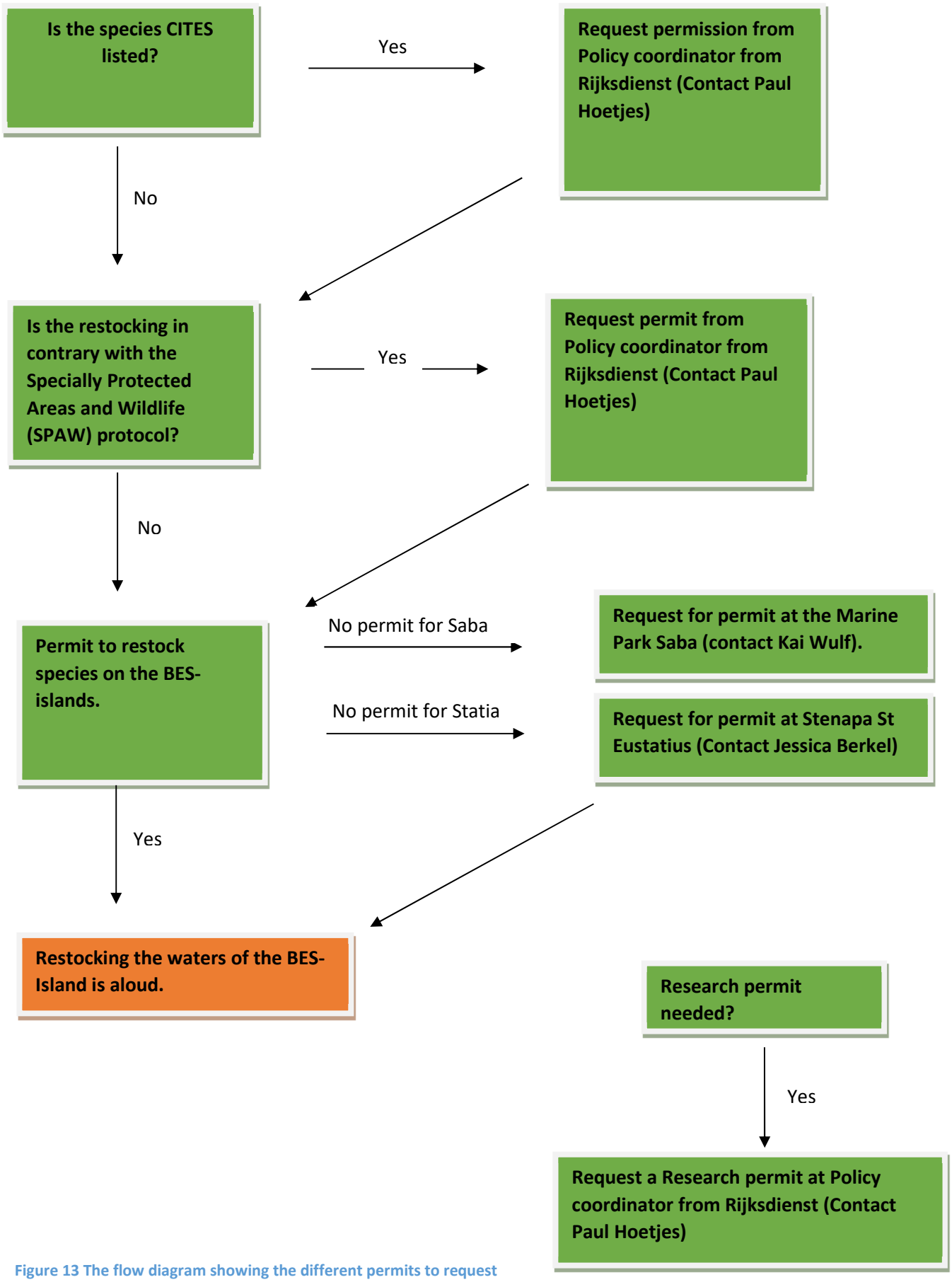


Figure 13 The flow diagram showing the different permits to request

The Marine parks have said that more scientific research is needed in order to set up a clear list of criteria that have to be met when applying for a permit. They will probably be using the mentioned before IUCN guidelines.

The Marine parks will have to determine whether the research will impact the ecosystem and to what level. Besides that, they will have to determine whether the research meets the goals of the nature conservation plans they have set up.

In the Nature Policy Plans for the BES-Islands it is stated that the nature parks in the Caribbean Netherlands have to develop management plans to achieve the general conservation goals. Both of the island have designated protection areas which make it illegal to restock wild populations of *D. antillarum* with cultured individuals (Figure 14 & 15). But the restocking of the *D. antillarum* could be a huge gain to their goals. It could overcome the algae problems on the reefs. With that help and the possibility of the communities on the reef thriving again the marine parks should decide if they can make a exception to restock *D. antillarum.*

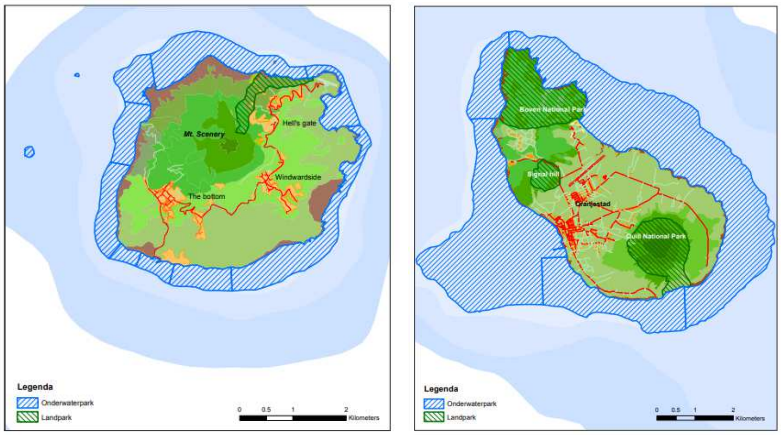


Figure 14 & 15 Saba (left) and Statia (right) and their protected areas, with the marine protected areas marked with blue stripes (Ministerie van Economische zaken, 2013)

To restock the reefs surrounding the islands, the exact same genetic species as existing on the reefs surrounding Saba and Statia today should be used. This can only be reached by using locally caught mature sea urchins. Importing *D. antillarum* doesn't prevent the risk of taking any diseases, and especially different genetically diversity to mix with the indigenous sea urchins.

The Marine parks will research whether the restock of the *D. antillarum* will impact the ecosystem and to what level. Besides that, they will determine whether the research meets the goals of the nature conservation plans they have set up. The problem at the moment is that the exact criteria that will have to meet are not clear. A possible reason for this might be because until now, nobody has ever researched this, so probably a list of criteria will be set up when the request for the permit is handed in according to Paul Hoetjes (Hoetjes, 2018).

7. Discussion

The Sea Urchin broodstock

D. antillarum is the main focus of this study. At first, 12 *D. antillarum* where purchased via De Jong Marinelife, who have imported the individuals from Cuba, as they have their own location on that Island. Whether these 12 individuals were mature or not has not been investigated, as in order to do so, the individuals have to be killed and taken apart and the gonads have to be examined (Benítez-Villalobos, et al., 2015). This was not possible due to the fact that the individuals were needed in order to obtain larvae. The process of obtaining larvae with shock treatment has been repeated multiple times, using the exact same technique as previous researchers have used (Moe, 2017; Moe, In Prep. 2017; Moe, In Prep. 2017(2)). This technique has been proven to be suitable to obtain larvae, but in this study did not turn out positive. Thus it was thought that the *D. antillarum* individuals were not mature yet.

Due to the fact that *D. antillarum* are not just for the taking in the Netherlands, a different approach had to be used. In this case, the nearby available *Diadema* species were located and purchased. In this case, only two *D. setosum* were available and thus used to obtain larvae. These individuals were considerably bigger compared to the *D. antillarum* individuals. Whether the larvae of both species have the exact same difficulties surviving has not been researched during this study, so future research is needed to determine whether the use of *D. setosum* larvae can be compared to the use of *D. antillarum* larvae. Almost immediately after placing the *D. setosum* in their aquarium, they started to spawn. The problem at that moment was that the larvae thanks that were ordered from Germany had not arrived yet, so there were no systems to keep the larvae in. The larvae were put in a simple aquarium, equipped with an air pump, but that was not sufficient as after one day, there were no alive larvae found in different samples.

Once the larvae tanks arrived, all systems were made ready and the spawning process started again. This time, only one of the two *D. setosum* started to spawn, most likely a female, as small eggs were discovered under a microscope. Since the process of obtaining larvae needs sperm and eggs, this was not sufficient enough and thus not used.

At this point, a decision had to be made on what to do next, as time started to run out. As can be read in the report, Brine shrimp (*Artemia salina*) are comparable to *D. antillarum* due to their pelagic life phase and size, their size is comparable to the size of the *D. antillarum* and *D. setosum* at an age of 25 to 35 days (Artemia international LLC, 2018; Moe, In Prep. 2017), so the decision was made to use *Artemia salina* as model organisms. But by using *Artemia salina*, questions may arise whether these organisms are sufficient enough to represent *D. antillarum* larvae. These questions are discussed below.

***Artemia salina* size**

As said in the problem description, *Artemia salina* are comparable to *D. antillarum* larvae at an age of 25 to 35 days. This means that a comparison is made between a culture of *Artemia salina* and a part of a culture of *D. antillarum.* The problem is that nothing can be said or concluded about the first 25 to 35 days of a *D. antillarum* culture, in which *D. antillarum* larvae are smaller in size, so it is questionable if a *Artemia salina* culture is representative enough for a full *D. antillarum* culture.

Salinity tolerance

Artemia salina is known to being able to tolerate high salinity levels (up to 340 ppt) (Mohammadi, et al., 2009), so a rise in salinity in the systems would not automatically result in higher mortality rates.

For *Diadema,* this is not the case, as research has shown that *D. setosum* larval survival decreased with increasing salinities, starting at a salinity of 31 ppt (Sarifudin, et al., 2017). For *D. antillarum,* this has never been researched, but due to the resemblance of *D. antillarum* and *D. setosum*, it can be assumed that this is the same for *D. antillarum*. This makes it hard to compare a culture of *Artemia salina* to a culture of *Diadema* as mortality levels are not the same with increasing salinities.

Temperature tolerance

Artemia salina is known to being able to tolerate large fluctuations in temperature (6 – 37 degrees Celsius) (Animal diversity Web, 2003), so a drop or a rise in temperature in the systems would not automatically result in mortality of *Artemia salina.* For *D. antillarum,* this is not the case. *D. antillarum* prefers temperatures between 4.28 and 27.8 degrees Celsius (EOL, 2016) and are not able to overcome higher temperatures, during which they lose the ability to righten themselves in case they fall over (Sherman, 2015). Besides that, a change in temperature in the natural ecosystem of the *D. antillarum* can result in the starvation of coral reefs which might eventually lead to *D. antillarum* losing their territory (Australian Government, 2018).

Water change

For the Kreisel tanks, changing an amount of water due to bad conditions of non-optimal parameters has been shown to be quite easy. Since the kreisel tanks were equipped with a mesh to enable water exchange with the interior and were placed in a larger aquarium, water change could be done by simply taking water out of the area outside the kreisel tanks themselves to prevent *Artemia salina* being taken out of the system. But for both the plastic bottles as for the beakers on the shaking table it was less easy. The air pump or the shaking table had to be shut down in order to let the *Artemia salina* sink to the bottom. This enabled the process of water change to be performed and minimizing the risk of taking *Artemia salina* out of the system. But since *Artemia salina* are able to move themselves, the risk of taking them out of the system by performing a water change never completely disappeared. This raises the question if the survival rate of these two systems has been calculated right, as the chance of individuals being taken out by accident is plausible. This could have been determined by checking the water that was taken out for any *Artemia salina.*

Higher survival rate of the Kreisel tanks

It is expected that the higher survival rate of the Kreisel tanks was caused due to the fact that the Kreisel tanks had a larger volume compared to the beakers and plastic bottles, which could also be seen at the nitrate levels, which were higher in the beakers and plastic bottles(Appendix I). Besides that, the Kreisel tanks enable a constant water change with the use of pumps, as where the plastic bottles and beakers are too small for this. Last but not least, the Kreisel tanks were easier to maintain and adjust, due to their size and volume.

8. Conclusion

8.1 *Artemia salina* experiment conclusion

As the results have shown, the plastic bottles had the lowest survival rate, followed by the beakers on the shaking table. The kreisel tanks had the best results by far with the highest survival rate and the highest mean amount of *Artemia salina* per ml after 13 days. Besides that, the Kreisel tanks are easier to maintain due of their size and water volume. From these results, the conclusion can be drawn that the Kreisel tanks had the highest survival rate, thus is the best system to use when culturing *Artemia salina.* But keep in mind, that despite the fact that the Kreisel tanks had the best results, the other two set ups also had positive results, which means that every set up is suitable for culturing *Artemia salina.*

8.2 Restocking policies conclusion

As the research has shown, it is possible to restock wild populations with cultured *D. antillarum* on the reefs surrounding the Dutch Caribbean Islands Saba and St. Eustatius, while complying to the current policies, but it is a very time consuming process, which needs more research to clarify the criteria that the Marine parks will set to determine whether a permit will be given or not. As said in the results section of this research: The Marine parks will have to research whether the introduction will impact the ecosystem and to what level. Besides that, they will have to determine whether the research meets the goals of the nature conservation plans they have set up.

9. Recommendations

While performing this research, we found that there is not a lot of data available about the population in regional areas. For example, it is only stated that there was a huge decline in the 1980s, but it is not possible to find any data about the local population in the water surrounding the Dutch Caribbean Islands Saba and St. Eustatius. To further research the possibilities to culture the *D. antillarum* on location and restock the wild populations, it is absolutely necessary to know more about the local populations at this moment. Also it is likely that this should be researched first, before the marine parks of both islands even handout a permit to restock the local waters. This is also seen in other restocking projects.

We also recommend that in future culturing of *D. antillarum,* the Kreisel tank should be used during the process, due to its positive effect on the growth of the larvae, but also due to the fairly easy handling and maintenance of this set up. The larger water volume of the Kreisel tank simplifies the process of keeping water parameters optimal, without the risk of damaging or taking out any larvae. These results come from a research that have used *Artemia salina* as a model organism, but it is expected that the Kreisel tank might have the same results for *D. antillarum*, except of course the increase in density of 445%, but extra research to determine whether the different set ups significantly differ from each other when using *D. antillarum* is recommended.

For future research it is recommended that bottlenecks when culturing *D. antillarum* are researched and inventoried, before trying to culture. It is believed that the brood stock that was used for this research was not mature yet, as the spawning process was done exactly the same as used in former research.

Some of the brood stock individuals suddenly died. It is still not determined what caused this, but it is believed that it had something to do with nitrate levels, as this was the only parameter in the system that was hard to control. Mainly low levels of nitrate were measured (0-10mg/L), with an exceptional peak in between (60 mg/L). Although former research states that nitrate does not have any negative effects of *D. antillarum* (Moe, In Prep. 2017), it has never been researched or tested what the exact effect was. Thus it is recommended that this is done in the future.

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Appendix I – Sampling data

This appendix shows all data that has been collected during the *Artemia* experiment per day. As for layout issues, the data tables start on the next page.

Date: 20-06	Number	Name	Litre	Algae per ml	Larvae amount	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	1374	30	8,1	24,8	0	0	0	0	0	0	Larvae added
	2	Kreisel	13,74	30000	1374	30	8,1	24,8	0	0	0	0	0	0	Larvae added
	3	Bottle	1	30000	100	30	8	24,6	0	0	0	0	0	0	Larvae added
	4	Bottle	1	30000	100	30	8	24,5	0	0	0	0	0	0	Larvae added
	5	Bottle	1	30000	100	30	7,9	24,5	0	0	0	0	0	0	Larvae added
	6	Bottle	1	30000	100	30	8	24,7	0	0	0	0	0	0	Larvae added
	7	beaker	0,5	30000	50	30	8,2	24,5	0	0	0	0	0	0	Larvae added
	8	beaker	0,5	30000	50	30	8,3	24,6	0	0	0	0	0	0	Larvae added
	9	beaker	0,5	30000	50	30	8,1	24,7	0	0	0	0	0	0	Larvae added
	10	beaker	0,5	30000	50	30	8,2	24,6	0	0	0	0	0	0	Larvae added

Larvae added by calculating the amount of larvae per 1 ml in the breeding setup. Then calculating it back to what is needed to fill all systems untill 0.1 larvae per ml is reached.

72,37ml kreisel, 5,26ml plastic bottle, beaker 2,63ml

Date: 21-06	Number	Name	Litre	Algae per ml	Larvae amount	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	1374	31	8,1	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	2	Kreisel	13,74	30000	1374	31	8,1	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	3	Bottle	1	30000	100	31	8	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	4	Bottle	1	30000	100	31	8	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	5	Bottle	1	30000	100	31	7,9	24,2	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	6	Bottle	1	30000	100	32	8	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	7	beaker	0,5	30000	50	31	8,2	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	8	beaker	0,5	30000	50	31	8,3	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	9	beaker	0,5	30000	50	31	8,1	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	10	beaker	0,5	30000	50	31	8,2	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 22-06	Number	Name	Litre	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	3160,2	2,3	31	8,1	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	2	Kreisel	13,74	30000	3847,2	2,8	31	8,1	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	3	Bottle	1	30000	300	3	32	8	25	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	4	Bottle	1	30000	260	2,6	31	8,1	24,8	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	5	Bottle	1	30000	250	2,5	31	7,9	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	6	Bottle	1	30000	270	2,7	31	8	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	7	beaker	0,5	30000	150	3	32	8	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	8	beaker	0,5	30000	140	2,8	32	8,3	24,6	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	9	beaker	0,5	30000	110	2,2	31	8,1	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	10	beaker	0,5	30000	120	2,4	31	8	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 25-06	Number	Name	Litre	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	4396,8	3,2	33	8,1	24,9	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	2	Kreisel	13,74	30000	4122	3	33	8,1	25	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	3	Bottle	1	30000	320	3,2	32	8	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	4	Bottle	1	30000	300	3	32	8	24,9	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	5	Bottle	1	30000	300	3	31	7,9	24,5	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	6	Bottle	1	30000	280	2,8	32	8	24,9	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	7	beaker	0,5	30000	150	3	34	8,2	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	8	beaker	0,5	30000	140	2,8	33	8,3	24,6	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	9	beaker	0,5	30000	110	2,2	32	8,2	24,9	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	10	beaker	0,5	30000	120	2,4	34	8,2	24,6	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 26-06

Number	Name	Litre	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
1	Kriesel	13,74	30000	4122	3	31	8	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
2	Kreisel	13,74	30000	4122	3	31	8	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
3	Bottle	1	30000	320	3,2	32	8	24,2	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
4	Bottle	1	30000	310	3,1	31	8	24,9	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
5	Bottle	1	30000	300	3	31	7,9	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
6	Bottle	1	30000	280	2,8	31	8	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
7	beaker	0,5	30000	150	3	31	8,2	24,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
8	beaker	0,5	30000	145	2,9	31	8,3	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
9	beaker	0,5	30000	120	2,4	31	8	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
10	beaker	0,5	30000	115	2,3	31	8,2	24,2	0	1	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 27-06

Number	Name	Total volume	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
1	Kriesel	13,74	30000	4671,6	3,4	31	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
2	Kreisel	13,74	30000	5770,8	4,2	31	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
3	Bottle	1	30000	200	2	32	8,2	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
4	Bottle	1	30000	200	2	32	8,2	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
5	Bottle	1	30000	200	2	31	8,3	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
6	Bottle	1	30000	190	1,9	32	8,2	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
7	beaker	0,5	30000	115	2,3	32	8,1	24,6	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
8	beaker	0,5	30000	120	2,4	31	8,1	23,5	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
9	beaker	0,5	30000	115	2,3	40	8	24	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
10	beaker	0,5	30000	115	2,3	32	8,2	24	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 28-06	Number	Name	Total volume	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	4946,4	3,6	31	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	2	Kreisel	13,74	30000	5770,8	4,2	31	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	3	Bottle	1	30000	200	2	31	8,2	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	4	Bottle	1	30000	210	2,1	31	8,2	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	5	Bottle	1	30000	200	2	31	8,3	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	6	Bottle	1	30000	190	1,9	31	8,2	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	7	beaker	0,5	30000	115	2,3	32	8,1	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	8	beaker	0,5	30000	120	2,4	31	8,1	23,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	9	beaker	0,5	30000	110	2,2	32	8	24	0	2	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	10	beaker	0,5	30000	115	2,3	31	8,2	23,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 29-06	Number	Name	Total volume	Algae per ml	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
	1	Kriesel	13,74	30000	6045,6	4,4	32	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	2	Kreisel	13,74	30000	5908,2	4,3	32	8,2	25	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	3	Bottle	1	30000	260	2,6	32	8,2	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	4	Bottle	1	30000	230	2,3	32	8,2	24,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	5	Bottle	1	30000	240	2,4	32	7,9	24,7	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	6	Bottle	1	30000	210	2,1	32	8	24,8	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	7	beaker	0,5	30000	140	2,8	32	8,1	24,6	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	8	beaker	0,5	30000	130	2,6	32	8,1	23,5	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	9	beaker	0,5	30000	150	3	32	8	24	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill
	10	beaker	0,5	30000	145	2,9	32	8,2	23,9	0	0	0	0	0	0	Larvae counted, osmosis added, algae calculation and refill

Date: 02-07	Number	Name	Total volume	Larvae per Litre	Larvae amount	larvae 10 ml	Salinity (ppt)	Ph	Temp	No2	No3	NH3/NH4	Po4	Ca	Mg	Additional information
Final results	1	Kriesel	13,74	460	6320,4	4,6	31	8,2	25	0	0	0	0	0	0	Larvae counted
	2	Kreisel	13,74	430	5908,2	4,3	30	8,2	25	0	0	0	0	0	0	Larvae counted
	3	Bottle	1	230	230	2,3	31	8,2	24,8	0	0	0	0	0	0	Larvae counted
	4	Bottle	1	240	240	2,4	31	8,2	24,9	0	0	0	0	0	0	Larvae counted
	5	Bottle	1	250	250	2,5	30	8,3	24,7	0	0	0	0	0	0	Larvae counted
	6	Bottle	1	220	220	2,2	31	8,2	24,8	0	0	0	0	0	0	Larvae counted
	7	beaker	0,5	290	145	2,9	31	8,1	24,6	0	2	0	0	0	0	Larvae counted
	8	beaker	0,5	280	140	2,8	31	8,1	23,5	0	1	0	0	0	0	Larvae counted
	9	beaker	0,5	310	155	3,1	31	8	24	0	2	0	0	0	0	Larvae counted
	10	beaker	0,5	320	160	3,2	30	8,2	23,9	0	1	0	0	0	0	Larvae counted

Appendix II – SPSS Output

Test of Homogeneity of Variances

SURVIVALRATE

Levene Statistic	df1	df2	Sig.
1,050	2	7	,399

Multiple Comparisons

Dependent Variable: SURVIVALRATE

Bonferroni

(I) FACTOR1	(J) FACTOR1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Kreiseltank	Plastic bottle	,21000*	,01445	,000	,1648	,2552
	Beakers	,14500*	,01445	,000	,0998	,1902
Plastic bottle	Kreiseltank	-,21000*	,01445	,000	-,2552	-,1648
	Beakers	-,06500*	,01180	,003	-,1019	-,0281
Beakers	Kreiseltank	-,14500*	,01445	,000	-,1902	-,0998
	Plastic bottle	,06500*	,01180	,003	,0281	,1019

*. The mean difference is significant at the 0.05 level.

ANOVA

SURVIVALRATE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,059	2	,029	105,646	,000
Within Groups	,002	7	,000		
Total	,061	9			