An integrated image of the effect of multiple stressors (temperature rise & plastic chemical pollution) on the harpacticoid copepod *Nitokra spinipes*

By

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Preface

The Flanders Marine Institute (Vlaams Instituut voor de Zee, VLIZ; Belgium) is a non-profit organization established in 1999 located in the province of West-Flanders. The institute focuses on marine research in the ocean, the sea, the coast and tidal systems. Their mission is: “to promote accumulation of marine knowledge and excellence in marine research”. VLIZ strives to reach this goal by keeping close connections with national and international research and educational institutes. To support their research, the knowledge institute features a wide range of equipment, varying from research vessels to sampling cores and underwater robots. The research framework of the knowledge institute consists of 6 main themes, namely the: Ocean services in a changing ocean, the Ocean Past, the Ocean Observation, the policy driven and responsive mode research, the Blue Sky research and lastly the Ocean and Human Health research theme (VLIZ, n.d.). Accordingly, the present research was performed within the Ocean and Human Health research unit of VLIZ.

Abstract

Since the Industrial Revolution, human activity has reinforced climate change to alarming rates and has introduced new stressors such as plastic pollution to the environment. Currently, 4.8 - 12.7 million tons of plastic litters enter the oceans every year. Globally alternatives to replace synthetic-based plastics are being developed, to find more sustainable solutions for materials and applications. Bio-based plastics, for example, form a major group of the newly introduced alternatives. However, the effect of bio-based plastics relative to synthetic-based plastics is poorly understood. Additionally, the potential impact of bio and synthetic-based plastics and their combined effect with other environmental stressors on aquatic life is rarely investigated. This leaves a knowledge gap in understanding the realistic effects of plastics in the environment. The goal of this study was to assess the combined effects of the leaching additives extracted from two polymers, bio and synthetic based plastics, combined with a temperature increase (+ 2 °C) on aquatic invertebrates. To do so, we used the brackish water copepod *Nitokra spinipes*, an important and relevant species in toxicity testing. In this study the toxicity of the bio-based plastic Poly Lactic Acid (PLA) and the synthetic-based plastic Polypropylene (PP) were examined. Additionally, a next generation trial test was included in this study to present a baseline for further testing of the possible effect of multiple stressors on the next generation of *N. spinipes*. In the test with PLA leachates a significant effect was measured of decreasing larval development rates (LDR) at increasing leachate concentrations. In the PP leachates test no significant effect of the applied stressor (PP) on the measured LDR was observed. In the multiple stressor scenario performed with PLA, a significantly lower LDR was observed compared to the treatments in which *N. spinipes* was exposed to only one stressor. This may indicate that *N. spinipes* was affected by the thermal stress indicating increasing vulnerability to additional stressors, such as plastic leachates of PLA. The multiple stressor scenario with PP leachate did not result in any observed delay in LDR’s after 7-days exposure. Further testing in the next generation trial experiment was therefore performed with PLA leachates. First results of this experiment indicate that PLA possesses chemicals that impact the spawning success of *N. spinipes* within the tested period of 2 weeks. No effects from the assessed stressors on the spawning trajectory, as well as the number of ovigerous females was found. Further testing to examine the exact extent of the effect of estrogenic activity (EA) exhibiting chemicals on the life cycle of *N. spinipes*, as well as research to other factors that may influence reproduction rates is still required. All together this study concludes that PLA, unlike PP, induces toxicity, affecting the survival of *N. spinipes* and that this effect is enhanced under a multiple stressor scenario.

Table of Contents

Chapter 1: Introduction

Problem statement

The oceans are under pressure and multiple stressors (e.g. global warming, pollution) can affect marine life. Currently, organisms are experiencing changes in the climate and their environment at unprecedented fast rates. This often creates stress and has the potential to make organisms increasingly vulnerable to additional stressors, e.g. synergistic or antagonistic effects (Portner, Langenbuch & Michaelidis, 2005). Plastic pollution is one of the most widespread environmental problems in the present time and oceans are filled with tons of poorly managed plastic waste each year (Parker, 2021). Plastic pollution can be a potential hazard to biota, as plastic pollution can harm marine organisms both physically and chemically. Macroplastics have the potential to cause physical entanglement this can cause among others suffocation and drowning leading to injuries and death (Encounter Edu, 2020; Wabnitz & Nichols, 2010). Microplastics can be ingested and potentially cause starvation among marine life. Additionally, exposure to additives of plastics via leaching may chemically harm marine life due to the toxic characteristics of some of the additives released in the water column (Capolupo et al., 2020). This can possibly contribute to the bioaccumulation of toxic chemicals in the food web (United States Environmental Protection Agency, 2020).

Chemical pollution is unlike physical entanglement, as it is not visible with the naked eye, but that does not make the problem any less impactful. De Frond et al. (2019) calculated that 7 of the most commonly found plastic objects in our oceans with a combined mass of 87,000 metric tons already account for 190 metric tons of chemical additives. These 7 objects included, according to De Frond et al. (2019): “Bottles, bottle caps, expanded polystyrene (EPS) containers, cutlery, grocery bags, food wrappers, and straws or stirrers”. Together these 7 objects are still only about 1% of the total amount of plastics entering the ocean each year(De Frond et al. (2019). In 2025, the mass of additives for the same 7 objects could already have risen to 370 metric tons, if plastic pollution continues to rise at current production and waste loss rates. Due to the persistent nature of numerous additives like Polychlorinated biphenyls (PCBs), they will continue to potentially induce toxicity to marine organisms. Chemical pollution may therefore be potentially more harmful than the physical particle (Bernes, 1998). There is still a major knowledge gap on the potential effects of major groups of additives used in the plastic industry, as well as the effect of mixture of these additives in the water column due to leachates (Campanale et al., 2020). Furthermore, the combined effects of climate change and induced chemical-toxicity via plastic leachates is currently not known. **We hypothesize that aquatic invertebrates that are subject to one stressor (temperature increase) will be more vulnerable to a second stressor (exposure to plastic leachates); and that the observed effects may cross to the following generation.**

Research focus

The broad goal of this project is to get an integrated image of the effect of multiple stressors on aquatic invertebrates and to fill knowledge gaps in this specific field of research. To do so, we expose the copepod *Nitokra spinipes* to control and increased temperatures (+ 2 °C) and plastic leachates, i.e. additives substances extracted from the matrices of synthetic and bio-based plastics into the water column. Copepods are the dominant form of zooplankton found in pelagic environments and are therefore an important and relevant species for toxicity testing. Their position in the food web connects the primary producers with the higher trophic levels. Besides their favorable position in the

food web, copepods are popular for toxicity testing purposes due to their distinct life stages, making development easy to track (Kwok et al., 2015).

The specific goal of this project is to assess the combined effects of temperature increase (+ 2 °C) and chemical leachates from synthetic and bio-based plastics on the copepod *N. spinipes*.

Accordingly, the main question of this research to obtain knowledge on the effect of multiple stressors on marine life is the following:

What is the combined effect of global warming (temperature, + 2 °C) and the exposure to leaching substances from plastics on the survival of the copepod Nitokra spinipes?

To answer this question, I divide the topic into the following subsections:

1. To what extent do leaching substances from bio-based plastics (e.g. Poly Lactic Acid (PLA)) alter the survival and development of the copepod *N. spinipes*?
2. How is this effect different from the impact of leaching substances from synthetic-based plastics (e.g. Polypropylene (PP))?
3. What are the single and the combined effects of raised temperatures and the exposure to leaching substances from plastics on the survival and development of the copepod *N. spinipes*?
4. To what extent is the next generation of the copepod *N. spinipes* affected by the single and the combined effect of raised temperatures and the exposure to leaching substances from plastics?

Chapter 2: Theoretical framework

Every year, 300 million tons of plastic are produced worldwide (Gourmelon, 2015). Plastic is found everywhere in our everyday life. From the computer we use for work, till the shampoo bottles in the shower, or the car we go on vacation with. Plastic is this popular due to its characteristics. Plastic has a great strength to weight ratio, is very durable, low in maintenance, cheap and resistant against corrosion. This makes plastic an economically favorable choice (BPF, n.d.).

Plastic does not just stay where we want it to stay, and can end up in the environment after being accidentally or inappropriately discarded. It can enter the oceans from a land-based source like: urban runoff, sewage overflow, industrial activities and many more. But the source of pollution can also be at sea itself. This is the case for lost material from aquaculture and fishery activities, as well for run off from ocean-based construction sides and other nautical activities (IUCN, 2018). Those sources together bring on a yearly basis an estimated amount of 4.8 till 12.7 million tons of plastic into our oceans (Jambeck et al., 2015).

Plastic entering our oceans comes in many forms and sizes. The term macroplastic is given to all plastic particles larger than 5 mm. This includes for example: bottles, fishing gear, plastic bags or straws. Most synthetic-based plastic debris do not biodegrade, but rather break down in smaller particles over time under the influence of UV radiation or due to mechanical stress (Monteiro et al., 2018). Particles from 1 micrometer till 5 millimeters are classified as microplastics (NOAA, n.d.). Microplastics are created in

the process of breakdown, but can also for example be produced for toiletry products. Spherically or amorphic shaped thermoplastics and thermoset plastics are added to the cosmetics to give the product the desired viscosity, skin conditioning or one of many other wanted features (Leslie, 2014). Via sewage systems they end up in the ocean and contribute to the estimated 15 till 51 trillion individual pieces of microplastics currently found in the oceans (Van Sebille, 2018). The specific size of microplastics makes them available via ingestion. Additionally, this size range is comparable to the prey size of many marine organisms and the plastic debris can therefore easily be mistaken for food (Galloway et al, 2017).

Plastic can be divided in two major groups: bio and synthetic-based plastics. Synthetic-based plastic is made from various materials, such as natural gas, coal, salt and the main ingredient, crude oil. The production of synthetic-based plastics starts off with the distillation of crude oil. The different boiling ranges of the oil divides the oil into groups of heavier and lighter oil components, that are known as fractions. A fraction consists of a mixture of hydrocarbon chains. These chemical compounds of carbon and hydrogen are each different in size and structure. During polymerisation and polycondensation, monomers are linked together to form longer chains, called polymers (Plastics Europe, n.d.). Bio-based plastics are made from an organic source that is naturally high in sugars, such as sugar beet, corn or wheats. For the example of wheats, the corns are milled into a substance called dextrose. This chemical substance is fermented in big vats by bacteria to lactic acid. The polymerization of lactic acid is inhibited by the production of water created by the linking of monomers to polymers. Therefore, the lactic acid molecules are linked prior to the main polymerization into smaller molecules called oligomers. The smaller amount of water that is released during this process is eliminated. Continuously the oligomers act as monomers that now can be polymerized into poly lactic acid (Ashter, 2016). The characteristics of the plastic is dependent on the monomers used for the formation of a polymer.

The characteristics of plastics are further modified by implementing additives into the polymer matrix. They are used as colourants, reinforcements, filters, or for functional purposes (Kontziampasis, 2012). Additives are also used to harden or soften a polymer in order to improve the final product (Banhegyi, 2012). Additives are different for bio and synthetic-based plastics (Innovative Industry, n.d.). In bio-based plastics natural substances as polyors and vegetable oils among others are mainly used, but also inorganic substances with low environmental impact, or easily biodegradable substances are used as alternatives to the conventional additives used in synthetic-based plastics (Samarth & Mahanwar, 2015).

Additives of plastic can leach into the waterbody as they release from the plastic medium by either external factors, such as weathering, mainly driven by radiation, or by mechanical influences. But also, the internal physical-chemical characteristics of the additives in the polymer matrix determines its leaching potency (Bejgarn et al., 2015). Additives with low hydrophobicity, a low octanol-water partition coefficient (Kow) or a low molecular weight are known to be more easily released into marine systems (Gunaalan et al., 2020). From some leachates, such as brominated flame retardants, their toxicity to the marine environment is already documented. But the effect of many individual leachates, as well as the mixture of leachates of additives into the water column is still unknown (Campanale et al., 2020)

Plastic pollution is not the only stressor marine organisms have to face in the present days. The climate is changing rapidly due to the accumulation of green-house gasses in the atmosphere and the green-house effect. The heating of the earth surface appears to go ten times faster than normal for ice-age recovery (Santer 1996). This is with a certainty of more than 95% due to human activity. The industrial revolution in 1760 was the start for the ongoing increase of anthropogenic carbon dioxide into the

atmosphere. Carbon dioxide is released in the atmosphere with a speed being 250 times faster than would be released from just natural sources. Due to carbon dioxide heat trapping characteristics radiation is not reflected straight back into the atmosphere, but gets partly trapped by the greenhouse gasses, absorbed and continuously reflected in all different directions, meaning as well back to the earth surface. This causes the temperature to rise (Department of Agriculture, Water and the Environment, 2020). The changing climate causes the ocean to heat up as well. Since 1969, the average temperature of the oceans has risen 0.33 °C (NASA, n.d.). Oceans store the excess heat, resulting from the increasing greenhouse gas levels in the atmosphere. The ocean works as a buffer zone protecting us from even faster climate change. As greenhouse gas emissions are expected to continue to rise, so will the temperature of the ocean. By 2100 the average temperature of the ocean is therefore estimated to rise between 1.5 and 2 °C (IPCC, 2019).

Rising temperatures are likely to induce stress on marine life (Hartmann et al., 2013). Heat stress is the phenomena that takes place when the temperature shifts outside the thermoneutral zone closer to the maximum critical temperature within a species thermal range (University of IOWA, n.d.). The organism gets into a state of hyperthermia. This negatively affects the metabolic rate of organisms, which translates into reduced growth and reproduction (Kroeker et al., 2013). A study of Breitholtz et al. (2003) on the effects of temperature rise on the copepod *N. spinipes*, discovered that increasing temperature slows down the development of *nauplii* to the adult stage and increases mortality within the population. Additionally, with rising temperatures the metabolic costs appeared to increase more rapidly than the assimilation. This decreases the efficiency of the metabolic process and leads to a decreased energy availability for reproduction. This results in a decreased number of offspring and smaller broods. Therefore, we expect in this study to find effects on the survival *of N. spinipes* related to temperature rise.

Background: The copepod *Nitokra spinipes*

The test organism, *Nitokra spinipes,* in this research is a copepod, belonging to the class of Maxillopoda. This is a group of small crustaceans, thriving in both benthic and pelagic systems. They are universally present due to their ability to survive a wide range of abiotic factors. Our specific test species, *N. spinipes,* for example has the ability to withstand salinity ranges from 0 till 30% and temperature fluctuations from 0 till 26 °C. Copepods make up the dominant form of zooplankton found in pelagic environments and are therefore an important link in the food web. Their position connects the primary produces with the higher trophic levels. This gives the unique opportunity to track and gain understanding on accumulation of toxic substances in the food web right from the start (Frangoulis et al., 2011). Besides their favorable position in the food web, the copepod is popular for toxicity testing purposes due to their distinct life stages, making development easy to track. The life cycle of a copepod consists of the egg stage, 6 naupliar stages (larvae), followed up by 5 copepodite stages (juveniles) and finally they reach adulthood (Devreker et al., 2004). For *N. spinipes* specifically, the lifecycle towards reaching sexual maturity takes 10 till 12 days under the ideal circumstances of a 22 °C temperature. Although the development towards adulthood takes only 10 to 12 days, the *N. spinipes* can live for several months if the environmental circumstances allow it (food availability, temperature etc.). The reproduction cycle of the *N. spinipes* is another reason why this species is popular for toxicity testing. This species has the ability to separate the fertilized eggs into individual egg sags. This allows researchers to precisely measure and track the reproduction effects and the possible effect of contaminants in toxicological testing (Kwok et al., 2015).

Chapter 3: Research design

To answer each of my scientific questions (chapter 1), I designed a specific set of experimental work (see appendix 2 for a visual overview of the experimental work for each experiment). All experimental work was based on a standardized ecotoxicology operating procedure (SOP) from ISO 18220 (ISO, 2016) (for the exact material list and method used in the experiments see appendix 3 & 4). Before running the first experiment, I underwent a period of training, to get familiar with the procedures for the toxicity test. Beneath an experimental design for each sub question is stated.

1. **Assess differences between exposure to leaching substances of synthetic versus bio-based plastics on the survival of the copepod *N. spinipes*:**

Leachates of synthetic (PP - polypropylene) and bio-based plastics (PLA - polylactic acid) were prepared in advance, prior to the exposure of *N. spinipes*. Plastics were kept in a medium solution for 3 weeks, in the dark, at 80 rpm and at room temperature (21 °C). In the end, I obtained a solution of leachates of unknown composition (blind test) at 100% concentrations for this specific period of time. The team at VLIZ, where I am collaborating with, will further explore the composition of these solutions, but this is out of the scope of my project.

Treatments: Leachate solutions, from PP and PLA, will be tested at a 100% concentration and the following diluted concentrations: 60%, 20%, 0%. Control (0%) solutions will be composed of medium (7 PSU filtered seawater) without any addition of leachates.

The following procedures ran over a one-week period. The activities performed per day are broadly described below.

- Day 0: For the experiment, I collected 50 adult gravid females per dish (5 dishes in total), separated them from the colony and kept them overnight for spawning purposes.

- Day 1: I collected +/- 10 no. of newly hatched *nauplii* per cell for testing purposes in the multiwell plate (6 cells per plate (see appendix 1)). Each cell was filled with 9 mL medium / dilution concentration and 1 mL *Rhodomonas salina* (microalgae, food). For each treatment I had 8 replicates, meaning that we used a total number of 32 test cells. This aligns with the use of 6 multiwell test plates. The multiwell plates were stored during the whole test in an incubator at 22 °C. This is the optimal temperature for development of *N. spinipes*.

- Day 2-7: (maintenance) - I fed the cultures 1 mL *R. salina* on the 3th and 5th day. On the same days I partially refreshed the cultures, executed tests (on parameters: conductivity, pH, survival, etc. to ensure validity) and I renewed the control solutions.

- Day 7: I counted the number of *nauplii* that have survived the experiment and noted down the number of *nauplii* that have developed into copepods over the test period for each treatment. I did this by observing the test cells with a microscope. This was done by two different observers for each replicate to ensure a valid outcome.

- Data analysis: For all tests the level of significance was stated at 0.05. For this test specifically, I assessed if there was a significant effect of exposure to the dilution series of 3-week leachates from PP and from PLA on the mortality and development rates of *N. spinipes*. To do this I performed a Kruskal-Wallis test, as the data did not meet the assumptions for ANOVA. Then, I compared if leachates from synthetic-based plastics are more toxic than bio-based plastic leachates or vice versa. From these results, I chose which set of variables I tested further under an increased temperature scenario. I selected the concentration where 50% of the *nauplii* did not develop into copepods (EC_{50}[1]) and tested it in the following experiments with increased temperature (i.e. 24 °C instead of 22 °C). The data analysis will be further developed by the team at VLIZ. In this study I will mostly discuss my results qualitatively (e.g. percentual increments).

2. Effects of rising temperatures and the exposure to leachates on the survival of *N. spinipes:*

I followed a modified version of the SOP for toxicity testing of plastic leachates, specifically designed for the pioneer testing of a multiple stressor scenario on the copepod *N. spinpes*. The additional stressor in this experiment was temperature rise. Therefore, the temperature tested within this experiment was 2 °C above the control temperature of 22 °C, based on the prediction of IPCC (2019) for 2100 (see introduction).

Treatments: We used a factorial design using the following treatments:

1) T0C0: 22 °C & control media (this is the control)
2) T0C1: 22 °C & selected leachate concentration based on experiment 1
3) T0C2: 22 °C & selected leachate concentration based on experiment 1 (in case of testing both plastic types)
4) T1C0: 24 °C & control media
5) T1C1: 24 °C & selected leachate concentration based on experiment 1
6) T1C2: 24 °C & selected leachate concentration based on experiment 1 (in case of testing both plastic types)

Acclimation period: 2 weeks prior to the test, I started acclimatizing 6 cultures of *N. spinipes* in an incubator over a 2 weeks period to 24 °C. I did this by increasing every 3^{th} /4^{th} day the temperature with 0.5 °C. The day prior to the test the *N. spinipes* was acclimatized to the increased temperature scenario and was fit for the making of precultures. This acclimation period is required for organisms to adjust to an increased temperature scenario, to avoid thermal shock.

The procedure followed ran over a one-week period. The activities performed per day are broadly described below.

- Day 0: For the experiment, I collected 50 adult gravid females per dish (3 dishes from the 22°C incubator and 3 dishes from the 24°C incubator). I separated them from the colony and kept them overnight for spawning purposes.

- Day 1: I collected +/- 10 no. of newly hatched *nauplii* per cell for testing purposes in the multiwell plate (6 cells per plate). Each cell was filled with 9 mL medium / dilution concentration based on experiment 1 and 1 mL *R. salina* (food). For each treatment I had 8 replicates, meaning that I used a total number of 48 test cells. This aligns with the use of 8 multiwell test plates. The test plates were split up in two groups: the T0 treatments and the

[1] For explanation *EC50 & *EC10 see appendix 4, method PLA/PP dilution series (page 38)

T1 treatments. To keep the temperature separate, treatment 1, 2 & 3 were randomized over the same 4 plates and treatment 4, 5 & 6 over the other 4 plates. The plates of T0 were stored during the whole test in an incubator at 22 °C and the plates of T1 in a second incubator at 24 °C.

- Day 2-7 were similar to the description of the previous experiment.

- Data analysis: For all tests the level of significance was stated at 0.05. For this test specifically, I tested if there was a significant effect of the exposure to multiple stressors on the development and mortality rates of *N. spinipes.* To do this an ANOVA test was performed, followed up by a Tukey test. Continuously I compared how these effects differed under the different treatments (described above). From these results I determined the single and combined effects of the stressors (temperature increase and exposure to plastic leachates) on the mortality and development of *N. spinipes*. The data analysis will be further elaborated by the team at VLIZ. In this study I will mostly discuss my results qualitatively (e.g. percentual increments).

3. Effects of rising temperature and the exposure to plastic leachates on the next generation of *N. spinipes* (Trial):

For this experiment the plastic type resulting in a significant effect on the mortality and/or development rates of *N. spinipes* based on experiment 1 & 2 was selected to assess the long-term effects of exposure to plastic leachates and temperature rise on the next generation of *N. spinipes*. In this experiment survival and reproduction success were the measurements of successful fulfillment of the life cycle. This experiment was conducted with the surviving organisms of the multiple stressor test. The reason for this was simply time management, most of the *nauplii* are likely to have developed into copepods or reached the end of the naupliar stages after one week of exposure to our selected treatments in the multiple stressor test. By partly merging the experiments I was therefore able to achieve results faster. At the end of the multiple stressor test, 2 replicates of each treatment were randomly merged, to achieve a total number of +/- 20 individuals in a test cell. I wanted a higher number of individuals per test cell during this next generation trial test to cancel out the chance of a significant difference in male/female ratios within our test groups. Due to the merging of cells I had 4 replicates instead of 8 for each treatment. For this specific test I followed a modified version of the method of Koch (Koch et al., 2017). I exposed the adults for 2 additional weeks to the experimental conditions after the multiple stressor test. During these two weeks the cells were closely monitored on the presence of ovigerous females. Ovigerous females were, when present, transferred from the 6-well plate to a separate 24-well plate in which the females were individually observed to track the spawning success in terms of brood interval time and the actual spawning of ovigerous females. This experiment differed from the previous two, as we conducted a full life cycle experiment instead of a larval development test. The purpose of this trial experiment was mainly to map the lifecycle of *N. spinipes* and to gather information for the designing of a protocol to enable optimization of future next generation toxicity experiments, as executing the full experiment is not within the time scope of my internship. This work will be continued by other colleagues.

The following procedures ran over a period of 21 days. The activities performed per day are broadly described below.

- Day 1: For the experiment I collected from the previous experiment +/- 20 adults per cell. Each cell was filled with 9 mL control medium / leachates dilution concentration and 1 mL *R. salina* (food). For each treatment I had 4 replicates. This aligns with 16 test cells. To optimize randomization, I divided the test cells over 4 6-multiwell plates. The test plates were again split up in two groups: the T0 treatments and the T1 treatments. To keep the temperatures, separate the T0 and T1 treatments were randomized within their own group of plates. The plates of T0 were stored during the whole test in an incubator at 22 °C and the plates of T1 in a second incubator at 24 °C.

- Day 2-14: (maintenance) Cultures were fed 1 mL *R. salina* every 1st, 3th, and 5th day of each test week (except from the first week, as the experiment starts on Friday this was on the 3th, 5th and 7th day). On the same day partial refreshment of the cultures, testing of parameters (conductivity, pH, survival, etc.) and renewing of the control solutions took place. Additionally, I checked every day if there were ovigerous females present in the cell. I noted this down per cell and transferred ovigerous females to a separate 24-well plate in which I monitored for each ovigerous female individually the brood interval time and eventually the spawning success. When *nauplii* occurred in the ovigerous female test cells, I transferred them to a separate *nauplii* 24-well plate to avoid errors that might occur otherwise if they had developed into copepods in the same cell as the ovigerous female.

- Day 14: On the 14th day of the next generation trial (the 21st day of their life cycle) I stopped monitoring the 6-well plates for the presence of ovigerous females. I continued to monitor the ovigerous female 24-well plates to map the brood interval time and the spawning success.

- Day 15 – (+/-) 21: The situation in the ovigerous female cells was monitored for one more week till the end of their life cycle. This was done to get a complete image of the possible effect exposure to plastic leachates and temperature rise may have on the lifecycle fulfillment of *N. spinipes.*

- Data analysis: I compared the spawning success in terms of number of ovigerous females and brood interval time of *N. spinipes* over the different treatment situations (selected based on experiment 1 & 2). From these results I determined the single and combined effects of the stressors: Temperature increase and exposure to leachates from plastics, on the successful fulfillment of the lifecycle of *N. spinipes*. The data analysis will be further elaborated by the team at VLIZ. In this study I will mostly discuss my results qualitatively and preliminary (e.g. percentual increments of measured effects).

Final remarks: All collected data together forms my framework to answer the main question of this research: *What is the combined effect of the global warming (temperature, + 2 °C) and the exposure to leaching substances from plastics on the survival of the copepod Nitokra spinipes?*

Open science

The data obtained from this study is stored in the marine data archive (MDA). This is an online platform for marine scientists founded by VLIZ. The MDA provides an archive with all collected metadata from experiments executed at VLIZ in a fully documented manner. It is used for managing data files and as personal back-up. Furthermore, it supports the transfer of knowledge between working groups and institutes, promoting more efficient research in the field of marine science (MDA, n.d.).

Chapter 4: Results

The toxicity larvae development blind test with PLA leachates

After exposing the *N. spinipes* larvae for 7 days to the leachates of Polylactic acid (PLA), our results indicate that PLA induced toxicity (Figure 1). The dilution series test with PLA showed a significant effect ($p_{Kruskal\text{-}Wallis}$ = $0.14*10^{-3}$) on the development of *N. spinipes*. By increasing PLA leachate concentrations, a decreasing percentage of *nauplii* that were able to develop into copepods was found. The highest average larval development ratio of 74% was found in the control (7 PSU diluted seawater). This is within the validity criteria of the ISO 18220: Dilution series blind test protocol (ISO, 2016) (as 60% of the individuals in the control +/- 20% should develop into copepods). This decreased at a 100% PLA leachate concentration to an average LDR of 2%, meaning that at this level of pollution the growth and development of the test individuals were inhibited almost completely. The EC_{50} of PLA leachate according to this test is located at 37% leachate (Figure 2). However, this is simplified for future test purposes to a 40% leachate concentration.

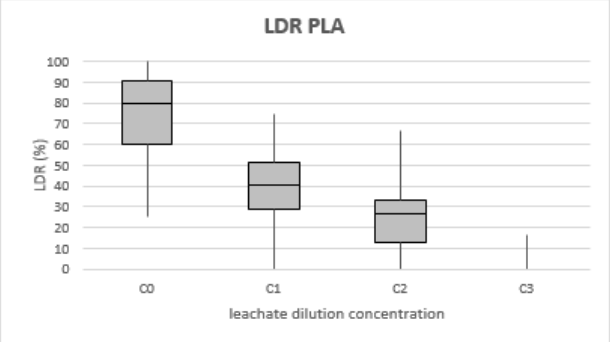

Figure 1. Boxplot of *Larval development ratio (LDR,%) against PLA dilution series (treatments)*

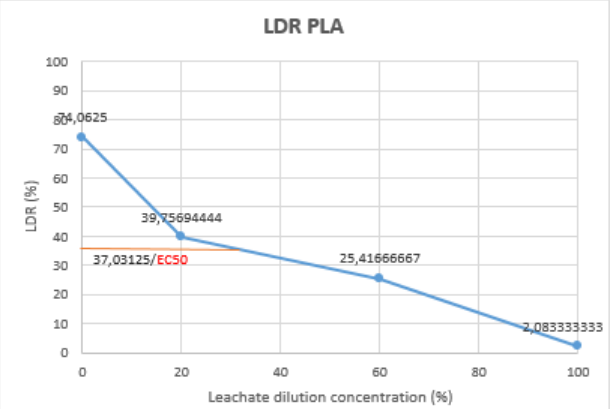

Figure 2. *LDR & EC50 against PLA Dilution series test (dilution relative concentrations)*

Mortality of the *N. spinipes* increased as well with increasing dilution concentrations (Figure 3.). The control showed on the day of observation a mortality of 13%. This is within the validity criteria set in the ISO protocol: Dilution series blind test (ISO, 2016). The highest mortality was found in the 100% PLA concentration, with a mean mortality of 35.79%. However, this was almost similar to the mortality observed at the 60% dilution concentration.

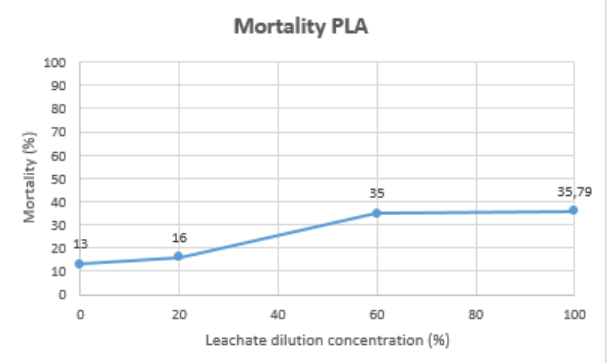

Figure 3. *Mortality rate (%) against PLA dilution series test (dilution relative concentrations)*

During the test week the parameters pH and conductivity were frequently measured in the cells labelled as “extra”; those cells did not contain *nauplii*, but only control media (Table 1.). The cells were refreshed on the same days as the cells containing the *nauplii*. Table 1 shows the values measured during the test. On day 3 and 5 the parameters are measured before and after renewal of the media, therefore the cell in the table is split up. The pH stayed during the entire test within 6 and 9. The conductivity varied during the test between 8 and 9. According to the ISO 18220 protocol: Dilution series blind test (ISO,2016) the conductivity may differ 10% from the start value. This would mean for this specific test a conductivity between 9.9 and 8.1. The refractometer used was not able to display decimals, but due to the small difference measured, the parameters in this test are considered to be valid.

Table 1. Validity parameters PLA dilution series

Parameter	Start of test	Renewal of medium				End of test
	Day 0	Day 3		Day 5		Day 7
pH	7.86	7.53	7.58	7.58	7.61	7.76
conductivity	9	9	9	8	9	8

The toxicity larvae development blind test with PP leachates

Seven-day exposure of *N. spinipes* larvae to the leachate dilution series (0, 20, 60, 100%) of polypropylene (PP) showed that there was no significant ($p_{Kruskal\text{-}Wallis}$ = 0.60) effect of leachate concentration in the degree of development in our test units (Figure 4 & 5).

The average Larval Development Rate (LDR) in the control was situated at 58.19%. This is again within the validity criteria stated by ISO 18220: Dilution series blind test protocol (ISO, 2016). This is as well the lowest average LDR observed during this toxicity test. In the test concentrations C0, C2 and C3 larval development rates of 100% were observed. The highest average LDR (71.88%) was observed in the C2 (60%) leachate concentration. The LDR did not show a pattern of induced effect by the leachate from PP.

The mortality rate (%) in the control exceeds the norm for validity (Figure 6). An average mortality of 37% was measured at the end of the test. According to the ISO 18220: Dilution series blind test protocol (ISO, 2016) this should stay beneath 20% for a valid test result. However, this test is still included in the report to compare in the discussion with fellow toxicity testing experiments of PP on diatoms and the multiple stressor test.

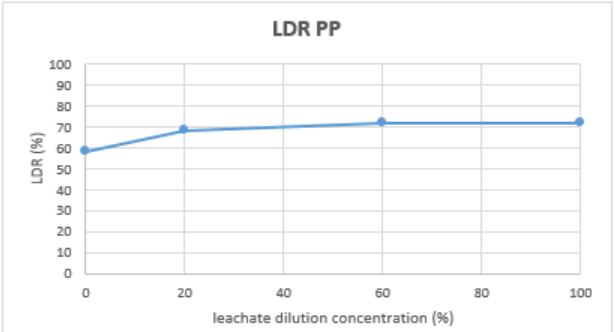

Figure 4. *LDR against PP Dilution series test (dilution relative concentrations)*

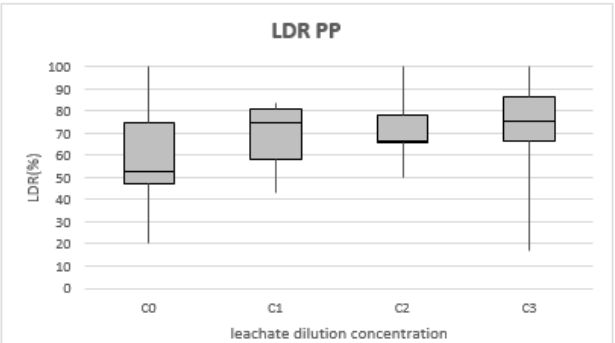

Figure 5. . Boxplot of *Larval development ratio (LDR,%) against PP dilution series (treatments)*

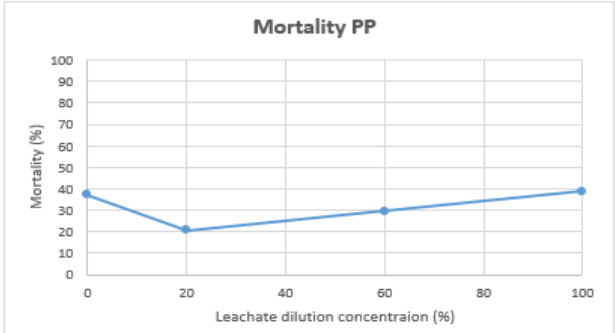

Figure 6. *Mortality rate (%) against PP dilution series test (dilution relative concentrations)*

The multiple stressor test with PLA

In this test *N. spinipes* was exposed for 7 days to four different stressor scenarios. This test resulted in a measurement of the single and combined effects of temperature rise and exposure to PLA leachates on the development rates, as well as the mortality rates of the copepod. In this test we used a 60% leachate concentration. This concentration is based on the multiple stressor trial test in which the *N. spinipes* was exposed to a 40% PLA leachate concentration. In this test the LDR after 7 days was too high to meet the validity terms, therefore in this test the concentration was increased to 60%, to slow down the process of development in order to measure a valid outcome.

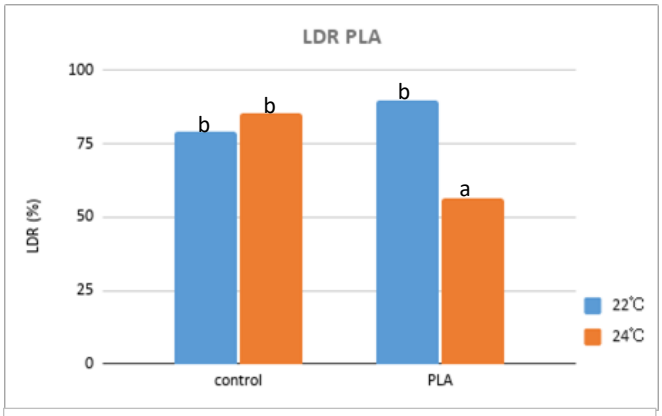

Figure 7. *Column graph of Larval Development ratio (LDR,%) against PLA Multiple stressor treatments. Bars with similar denotations did not differ significantly from each other (p=0.05)*

The control in this test is the treatment T0C0, in which no stressors were applied. In Figures 7 & 8 the average LDR measured over the different treatments is shown. The average LDR measured in the control is 79.0%. This is within the validity criteria of ISO 18220: Multiple stressor protocol (ISO, 2016). The highest average LDR (89.6%) was observed for the treatment T0C1. In this treatment the organisms were exposed to a singular stressor: exposure to 60% PLA leachates. The lowest average LDR (56.4%) was observed at T1C1. This treatment contained both stressors. This is the only treatment in which a significant difference relative to the other treatments is measured ($p_{ANOVA} \leq 0.15*10^{-1}$).

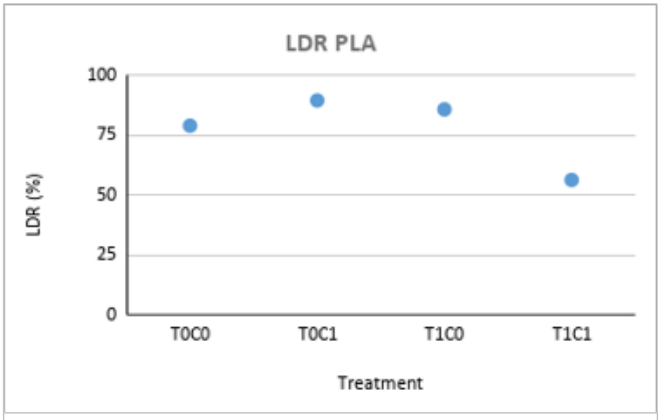

Figure 8. *Larval Development ratio (LDR,%) against PLA Multiple stressor treatments.*

Figure 9 shows the distribution of LDR's observed within the treatments. This indicates that in all treatments except for T1C1 LDR's of 100% were observed. The lowest LDR (28.6%) was observed in the treatment T1C1.

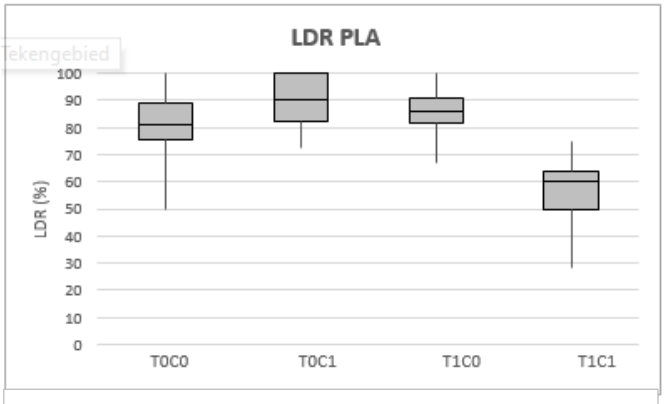

Figure 9. Boxplot of *Larval development ratio (LDR,%) against PLA Multiple stressor treatments.*

The mortality of *N. spinipes* increased with increasing temperature rather than the presence or absence of PLA leachates, according to this test (Figure 10). The lowest average mortality (9.0%) was observed in the control T0C0. This is within the validity terms of ISO: Multiple stressor protocol (ISO, 2016). The mortality increases up till 27.4% for the treatment T1C0 but decreases again to 20.9% at T1C1.

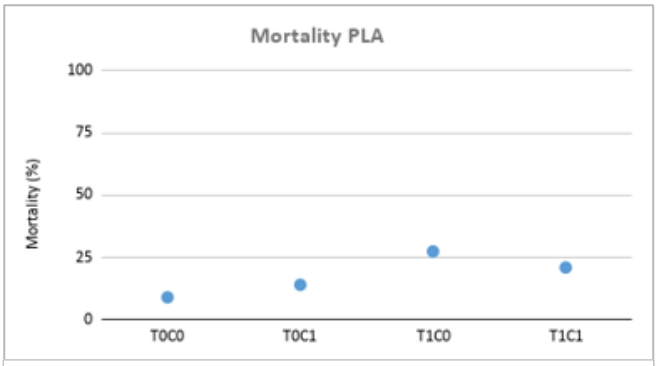

Figure 10. *Mortality rate (%) against PLA Multiple stressor treatments).*

During the test week the parameters pH and conductivity were frequently measured again to check the validity criteria of ISO 18220: Multiple stressor protocol (ISO, 2016) (table 2 & 3). This was done separately for T0 and T1, as different control media for the test groups was used to maintain the desired temperature. For both treatments the parameter pH stayed between 6 and 9. The conductivity varied less than 10% for both treatments as well. The test is therefore considered to be valid.

Table 2. Validity parameters PLA Multiple stressor test T0C0 (control treatment: 22°C, no leachates)

Parameter	Start of test	Renewal of medium				End of test
Treatment T0C0	Day 0	Day 3		Day 5		Day 7
pH	7.35	7.42	7.25	7.38	7.40	7.28
conductivity	7	7	7	7	7	7

Table 3. Validity parameters PLA Multiple stressor test T1C0 (24°C no leachates)

Parameter	Start of test	Renewal of medium				End of test
Treatment T1C0	Day 0	Day 3		Day 5		Day 7
pH	7.45	7.43	7.43	7.37	7.57	7.77
conductivity	6	6	6	7	7	7

The multiple stressor test with PP

In this test, *N. spinipes* was exposed for 7 days to four different stressor scenarios. However, this time the effects of exposure to PP leachates on the development and mortality rates of the copepod was tested. In this test we used a leachate concentration of 60%. The control groups in this test are the same as for the prior described test with PLA, as they were executed in the same experiment. This means that for this test as well all validity criteria according to ISO (2016) were met.

The highest average LDR measured in this test was again found for the treatment T0C1 (Figure 11 & 12). This time with an average LDR of 92.7%. The lowest average LDR of 77.0% was found in the treatment T1C1. According to this test neither exposure to PP leachates nor inducing temperature stress inhibited the development significant relative to the control ($p_{Tukey} \geq 0.16$).

The boxplot (Figure 13) shows the distribution of LDR's measured over the different treatments. In all treatments LDR's of 100% were observed. The standard deviation of T0C1 is the smallest and together with the high LDR's observed in this treatment. It may indicate that 60% PP leachate at 22 °C positively influences the development of *N. spinpes*.

The mortality of *N. spinipes* in this experiment was enhanced in the treatments with the stressor temperature rise and in the absence or presence of PP leachates (Figure 14). The lowest average mortality rate (9%) was observed at T0C0, in which no stressors were applied. The average mortality rate increases up to 27.4% by increasing the temperature with 2 °C for the treatment T1C0. The fluctuations in mortality rates between C0 and C1 treatments occur to not be significant (p_{ANOVA} = 0.89) according to this test.

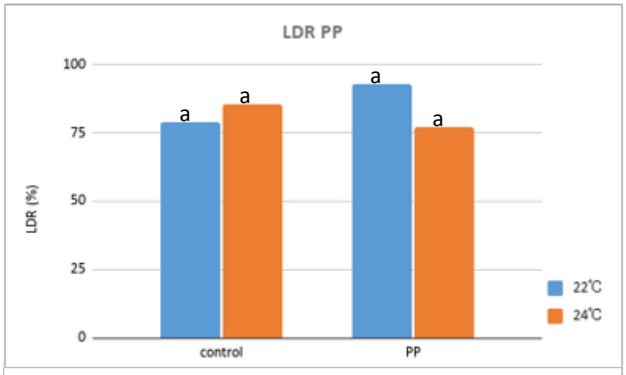

Figure 11. *Column graph of Larval Development ratio (LDR,%) against PP Multiple stressor treatments. Bars with similar denotations did not differ significantly from each other (p=0.05)*

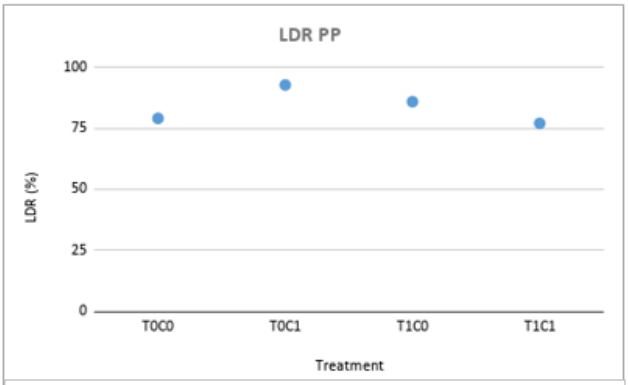

Figure 12. *Larval Development ratio (LDR,%) against PP Multiple stressor treatments*

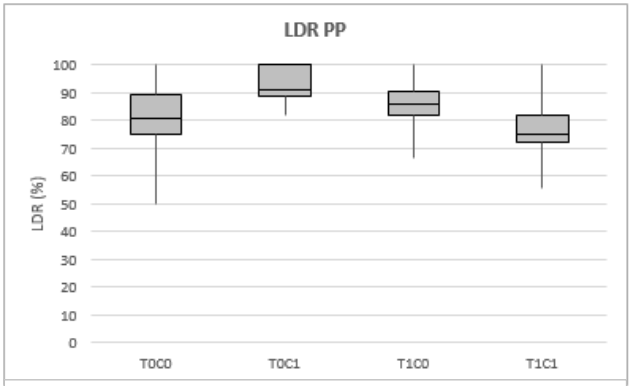

Figure 13. Boxplot of *Larval development ratio (LDR,%) against PP Multiple stressor treatments.*

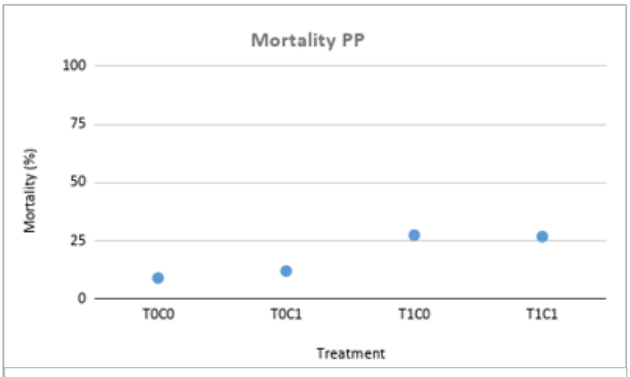

Figure 14. *Mortality rate (%) against PP Multiple stressor treatments.*

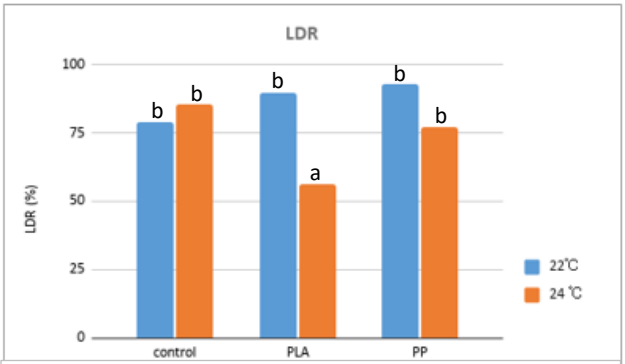

Figure 15. *Column graph of Larval Development ratio (LDR,%) against PP & PLA Multiple stressor treatments. Bars with similar denotations did not differ significantly from each other (p=0.05).*

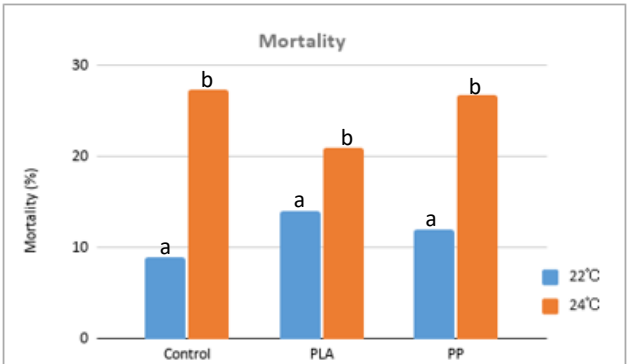

Figure 16. *Mortality rate (%) against PP & PLA Multiple stressor treatments. Bars with similar denotations did not differ significantly from each other (p=0.05).*

In Figure 15 the LDR's of the control, PLA and PP are shown. This figure emphasizes the difference in LDR's caused by the increased temperature. According to our tests only for PLA at T1 this resulted in a significant difference ($p_{ANOVA} \leq 0.15*10^{-1}$) compared to the other treatments. All treatments including temperature rise (24 °C) resulted in higher mortality rates after 7-day exposure to this stressor (Figure 16). In the control the mortality rose from 9 till 27.4%. In the treatments containing plastic leachates this difference was a little less extreme, but still a significant ($p_{ANOVA} = 0.61*10^{-3}$) effect of enhanced mortality at the increased temperature scenario was observed.

Next generation trial test

This test was executed as a follow up on the multiple stressor trial test with a 40% PLA leachate concentration. In the multiple stressor trial, we encountered too high LDR's in the control to include this test in the report. However, this test was still usable for further testing purposes in the next generation trial experiment. This trial experiment is meant to map the life cycle of the copepod *N. spinipes*, to create a protocol for future testing and to measure deviations in reproduction success between the different treatments. For the protocol written based on this experiment see appendix 4.

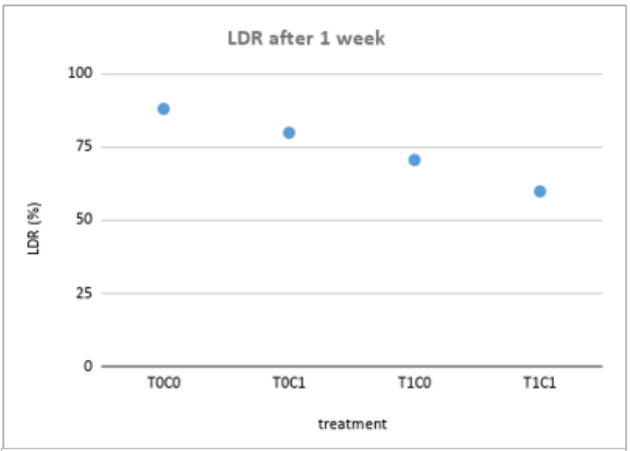

Figure 17. *Larval Development ratio (LDR,%) against PLA Multiple stressor treatments (1 week exposure in multiple stressor trial test (40% PLA)).*

After the multiple stressors trial test, the average LDR's observed are shown in figure 17. In T0C0 90% of the larvae were developed into copepods after a one- week period. This gradually decreased to an average LDR of 60% in T1C1.

Figure 18 shows the gradient of increase in the number of ovigerous females per treatment over a 2-week period (additional to the first week in the multiple stressor trial). On the 6th day of the next generation test (the 13th day of their actual life cycle) the first ovigerous females occurred in the T1C1 treatment. This doubled on the 7th day and created a great difference compared to the other treatments, where the first ovigerous females just started to occur. In T1C1 the largest increase in ovigerous females was at an earlier stage in the life cycle compared to treatment T0C1 and T0C0 in which ovigerous females occurred more constantly at a lower rate during their lifecycle. In T1C0 a total of 5 ovigerous females was found after the 3th week of their lifecycle.

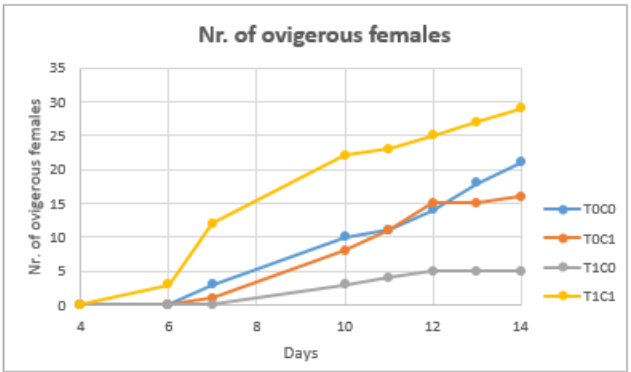

Figure 18. *Ovigerous females (no.) against days, per Multiple stressor treatment.*

For T0C1 and T1C1 there is an uncertainty, as shown in figure 19. During the weekend a number of females became ovigerous and spawned in the 6-well test plates. Based on the number of *nauplii* and the average occurrence of ovigerous females per day this uncertainty was estimated on 3 extra ovigerous females for both treatments. According to this correction the difference in number of ovigerous females between T0C1 and T0C0 is negligible.

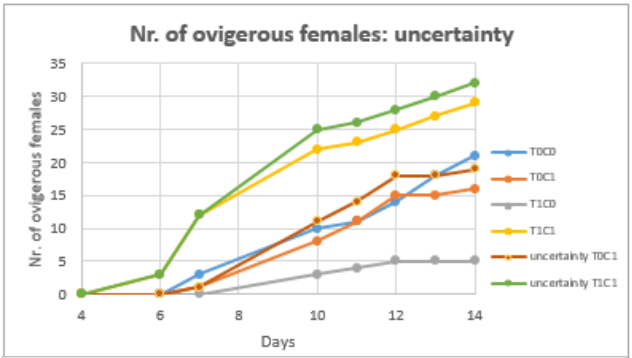

Figure 19. *Ovigerous females (no.)against days, per Multiple stressor treatment-uncertainty.*

In Figure 20 the total number of ovigerous females after 3-weeks per treatment is shown. The highest percentage of ovigerous females per treatment was found at the treatment T1C1. This treatment reached an average amount of ovigerous females in the test cells of 45.1%. The second-best treatment was T0C0 in which no stressors were applied, with 28% ovigerous females in the population. This was closely followed by T0C1 with 24%. T1C0 had the lowest outcome with only 7% ovigerous females in the population.

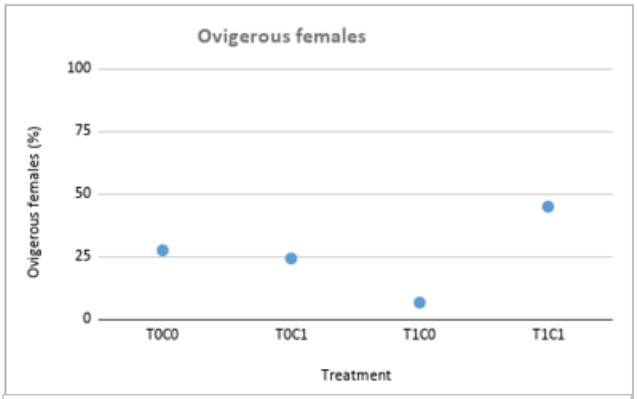

Figure 20. *Ovigerous females (%) against Multiple stressor treatments, after 3-weeks exposure.*

The spawning trajectory of the ovigerous females separated from the population for individual monitoring is visualized in figure 21. The first ovigerous females spawned on the 6th day of the next generation test (13th day of their actual lifecycle). The ovigerous females were monitored till the 21st (28th day of their actual lifecycle). Figure 21 shows the number of females that spawned over the test period in days. Most females spawned in treatment T1C1. This is the treatment with the most ovigerous females (figure 20). After the 16th day of the test (23rd day of the actual life cycle) the number of spawning females stabilized in all treatments. In T1C0, no females spawned.

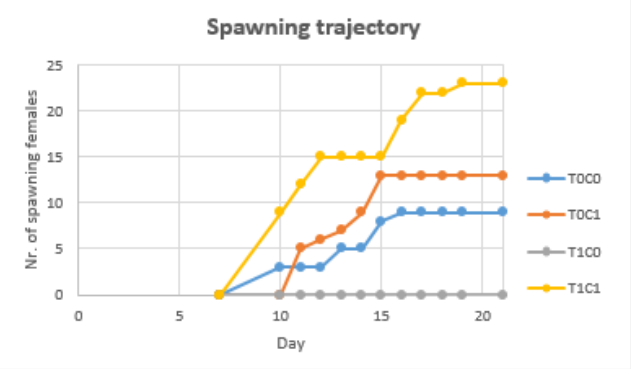

Figure 21. *Spawning trajectory (no. spawning females) against days per Multiple stressor treatment.*

In figure 22 the data from the spawning trajectory is corrected by the number of ovigerous females to calculate the spawning success of the ovigerous females. This is the percentage of ovigerous females that actually spawned *nauplii* over the entire lifecycle test (28 days). The spawning success was the highest in the treatments containing the 40% leachate dilution concentration. Both C1 treatments reached a spawning success of 80% (+/-1%). This is twice as high as the spawning success observed in T0C0, which was 43% after 28 days. In T1C0 the ovigerous females died before spawning or were unable to produce alive *nauplii*.

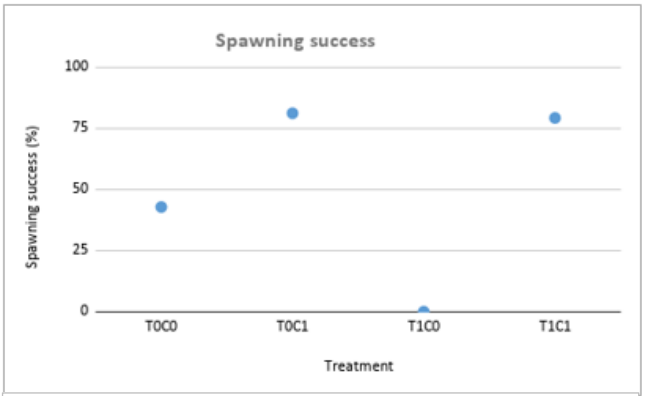

Figure 22. *Spawning success (%) against Multiple stressor treatments.*

The mortality observed in the treatments over the 28 days next generation test was for all treatments below 10% (Figure 23). The highest mortality was found in treatment T1C0. This was also the treatment with the lowest number of ovigerous females and the lowest spawning success. For the other treatments the mortality was either close to or similar to 0.

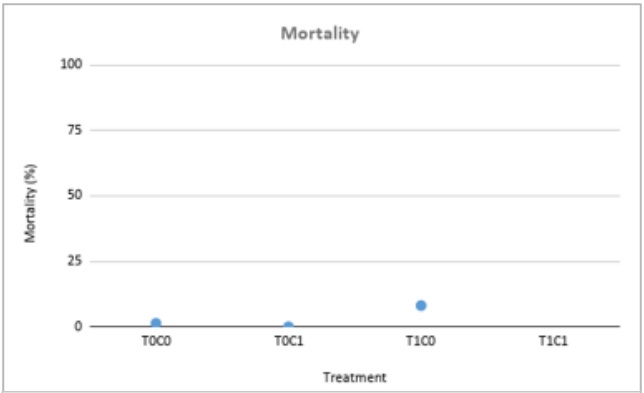

Figure 23. *Mortality rate (%) against Multiple stressor treatments.*

During the test period the parameters pH and conductivity were frequently measured again to check the validity criteria of ISO 18220: Next generation test protocol (ISO, 2016) (table 3&4). This was done separately for T0 and T1, as different control media for the test groups was used to maintain the desired temperature. For both treatments the parameter pH stayed between 6 and 9. The conductivity varied less than 10% for both treatments as well. The test is therefore considered to be valid.

Table 4. Validity parameters Next generation test T0C0 (control treatment: 22°C no leachates)

T0C0	Parameter											
	pH	7.48	7.62	7.58	7.64	7.60	7.53	7.47	7.67	7.65	7.43	7.44
	conductivity	7	8	8	8	7	7	7	8	8	7	8

7.56	7.54	7.56	7.55
7	7	7	8

Table 5. Validity parameters Next generation test T1C0 (24°C no leachates)

T1C0	Parameter											
	pH	7.49	7.73	7.64	7.76	7.51	7.81	7.56	7.76	7.70	7.58	7.56
	conductivity	7	7	7	8	7	7	8	7	7	7	7

7.79	7.77	7.67	7.60
8	7	8	7

Chapter 5: Discussion

PP & PLA dilution series

In this study we observed that toxicity testing with PLA and PP in the dilution series test resulted in significantly different responses in our test groups. The *N. spinipes* larvae exposed to PLA showed a clear response between increasing leachate concentrations and delay in the grade of development, while for PP the observed development pattern occurred to be rather random. This could be explained by the fact that bio-based plastics, such as PLA, potentially releases more easily additives in the water column due to the biodegradable nature of the substance, however this remains to be verified in our samples. For example, the expert Arthur Huang (Fairs, 2019) supports the theory that biodegradable plastics such as PLA are potentially more harmful to the environment than conventional plastics because they are designed to break down. This has as consequence that the substance reacts with the environment it is released in, as PLA is an acid it can potentially change the pH of the water creating a chemical problem. When comparing the pH of 21-day PP with 21-day PLA leachate a small but yet visible decrease in pH is indeed measured: 7.84 for PP and 7.64 for PLA. However, this difference in pH is too small to explain on its own the extreme decline in developmental rates in the PLA test groups compared to those exposed to PP leachates.

Another possible explanation for the increased toxicity induced by PLA can be found in the processing of the bio-based plastic. Tabone et al. (2010) found that for the growing of the crops used for the formation of PLA various pesticides and fertilizers are used that may cause the release of pollutants in the environment during biodegradation. This is likely to enhance the toxicity of PLA. This is in line with observations we made during the preparation of the leachate dilution series. Prior to the making of such a series, the leachate is filtered through a 0.2 micrometer filter, we experienced during this process that for PLA filtration we needed to change the filter 3 times as often than for PP filtration. This may indicate an increased amount of particles in the fluid.

Polypropylene (PP), the reference synthetic-based plastic used in this research, is known to be non-react. It is designed for long-term duration and therefore it can potentially release less easily chemical substances to the water column according to Arthur Huang (Fairs, 2019). This may explain why 21-day leachate of PP does not (yet) seem to induce toxicity on *N. spinpes* larvae (*nauplii*).

In a similar research of Z. Niu et al. (unpublished) on the effect of PP and PLA leachates on the specific growth rate of diatoms, an alike outcome was observed. A test with PLA resulted in an EC_{10}[2] value of 66.7% ± 15.9%, while for PP the EC_{10} was found at a leachate concentration of > 100%. This supports the finding in this research that PLA induces greater toxicity within the tested exposure period than PP.

The results of the PP test executed are preliminary, as according to the ISO 18220: (ISO, 2016) the validity terms were not met. This was due to the mortality rate of 37% in the control, which exceeded the allowed 20% limit. This high mortality rate is most likely caused by a so-called blunder in the maintenance process. During the maintenance procedure 7 mL of liquid is taken out of the test cell with a pipette and the cell is refilled with 6 mL new leachate and 1 mL algae food. During the process of removing liquid, *N. spinipes* may have been accidentally transferred as well. This can result in a false high mortality rate in the cell. This is most likely the case, as comparison with the data of the PLA test indicates that the control normally represents the lowest mortality rate. Although the test results are

[2] For explanation EC50 & EC10 see appendix 4, method PLA/PP dilution series (page 38)

still preliminary they do give a strong indication that PP leachates have had no effect on the LDR of *N. spinipes* within this experiment.

Multiple stressor test

In the multiple stressor test, we observed a significant delay in growth when exposing the organism to both PLA leachates and raised temperature . The results also indicate that adding the raised temperature to the test made the organisms more vulnerable to the presence of PLA leachates (figure 7). This is in line with a study of Heugens et al. (2001) on the effects of multiple stressors (including chemical stress from plastic pollution and climate change) on aquatic organisms in general. From this research it was concluded that organisms exposed to raised temperature close to the edges of their thermal range, appeared to be more vulnerable to additional stress of chemical pollution.

In the multiple stressor test with PP leachates there was again no significant effect measured of induced toxicity on the LDR of *N.spinipes* caused by the presence of PP leachates. This supports the findings of the previous dilution series test.

In all tested treatments including both the PLA and PP multiple stressor test, there was a significant rise in mortality rate observed at the increased temperature scenario. This is in accordance with the findings in a study of Koch et al. (2017). In which they exposed *N. spinipes* to different climate scenarios as well. In this study they tested the mortality rates at 20 & 25 °C. At 20 °C the mortality within the population was 4% this increased up till 17% at 25 °C. This research supports the findings of our experiment that temperature rise effects the mortality rates of *N.spinipes*. However, in our experiment the mortality rate in T1C0 was 27.4%, 10% higher than observed in a similar test at 25 °C. This may be potentially due to genetic differences between our test population and the test population used by Koch et al. (2017) or due to the different preparation technique of control media used by Koch et al.(2017). The control media in their experiment was besides filtered, also heated up till 80 °C to eliminate unwanted factors that may influence mortality rates.

The multiple stressor test was performed with a 60% leachate concentration. This was unlike our goal in the experimental design to use the EC_{50} value. However, testing with 40% leachates as performed in the multiple stressor trial test resulted in too high LDR's within the 7-day test period to consider the test valid. A possible explanation for this difference in development of *N. spinipes* between similar experiments may have nothing to do with their reaction on the leachate concentration, but rather on the improvement of other conditions the organisms were exposed to, like the quality of the algae food they received.

The quality of the algae food, that we used for the experiments, has differed between the test weeks. This was due to contamination of the algae culture, which resulted in some weeks (including the test week of the PLA dilution series, from which we obtained the EC_{50} value) in a reduced water quality and a low nutritional value. According to Ederington et al. (1995) the quality of the feed heavily influences the development of copepods, which may explain why the EC_{50} in the test week of PLA dilution series was found at a rather low leachate concentration unlike the EC_{50} value observed in the multiple stressor trial test. Based on the multiple stressor trial test we increased therefore the test concentration to 60% leachates. In the PLA dilution series (Figure 2) an average LDR of 25.42% at 60% leachate concentration was observed. This test showed a clear effect of induced toxicity by PLA. However, in the multiple stressor test (Figure 8) at 60% leachates the average LDR measured was 89.58% and showed no significant difference relative to the control group. This may again be caused by the improvements we made in our maintenance of algae cultures. This improvement may have

canceled out the delay in growth on the 7th day that we could observe previously in the PLA dilution series test. This may explain why we were only able to observe an effect for the treatment with both PLA leachates and temperature rise in the multiple stressor test. The organisms in this experiment were affected stronger due to the combined stressors and just improving the algae quality could not cancel out the delay in development on the 7th day.

In the multiple stressor test with PP leachates we observed higher average LDR's for each treatment as well, compared to the dilution series test with PP in figure 4. This is most likely caused by the improvement in algae food as well. In the dilution series test at 60% leachate concentration we initially found an average LDR of 71.88%. In the multiple stressor test this was for the same concentration at the same temperature 92.68%. The organisms exposed to temperature stress in this experiment did not show a significant difference in growth rates relative to the control group. This might be due to the overall high LDR's found in this experiment caused most likely by the improved algae feed quality that can cancel out a visual delay in LDR's at the 7th day of the test.

Next generation trial test

The next generation trial test was performed with a 40% PLA leachate concentration. This was the EC_{50} obtained from the PLA dilution series test. In this test exposure to the multiple stressor treatments resulted in a gradual decline in development rates by increasing the number of stressors. However, even though the exposure to multiple stressors initially delayed the grade of development in the test groups, the presence of PLA leachate rather than the temperature stressor appeared to enhance the spawning success in the end of the organism's life cycle.

In a study of Yang et al. (2011) plastic types including PLA were scanned on the presence of chemicals that exhibit estrogenic activity (EA). According to this test 70 up till 100% (depending on the extraction solvent used) of the tested PLA samples contained EA inducing chemicals. Those chemicals have the ability to mimic the naturally present hormone estrogen. EA exhibiting chemicals can bind with the hormone receptor of estrogen, this allows the chemical to disrupt the endocrine system and transmit altered signals to the body. This may cause several problems in the developmental and reproductive stages of organisms, such as altered sex-ratio's within populations, the under or over development of sex organs and early puberty resulting in premature pregnancies (Della Seta et al., 2006). This might explain the higher percentage of spawning success observed for ovigerous females exposed to PLA leachates within two weeks. The exposure to PLA leachate containing the endocrine disrupting chemicals may have cut short the gestation period of the ovigerous females as well, which would explain why the spawning success for those females was around 80% in the two-week observation period, while for the ovigerous females in the control this was only 40%.

The number of ovigerous females found among the treatments did not show a significant effect resulting from any of the stressors the organisms were exposed to. More research in next generation testing including multiple stressor scenario's is necessary to better understand possible factors (if any) that may influence reproduction rates.

Chapter 6: Conclusion

The larval development blind test with exposure to the singular stressors: 21-day PP & PLA leachates, concluded that within the tested period (7 days exposure) PLA rather than PP induced toxicity, resulting in a delay in the development of *N. spinipes*. A significant effect of increasing PLA leachate concentrations on decreasing LDR's was found. The lowest larvae development rate (0%) were observed in the test groups exposed to 100% PLA leachates. This research concludes that exposure to PLA in their environment may chemically affect the survival of *N. spinipes*. The exposure to PP leachates did not result in a negative response in LDR's over the tested period. No significant effect of increasing PP leachates on any measured effect was found.

Exposing *N. spinipes* to the multiple stressor scenarios resulted in a significantly lower average LDR in the treatment including both PLA leachates and temperature rise. This test indicated a higher vulnerability of the organism when exposed to two stressors and supports the hypothesis of this research in the case of PLA. The multiple stressor scenario with PP did not result in any significant delay in development. This outcome is in line with the previously performed PP dilution series test and indicates that PP in this experiment was not a stressor that has any negative effect on the development of *N. spinipes*.

The mortality in both multiple stressor tests increased in the increased temperature scenario. This is in line with a study of Koch et al. (2017).

Further testing with PLA leachates in a multiple stressor next generation test indicated an enhanced spawning success in the test groups exposed to PLA leachates. From this experiment, we concluded that the chemicals in PLA induce toxicity and may influence the gestation period of the ovigerous females. The spawning trajectory and the number of ovigerous females per treatment was not affected by the stressors measured in this experiment.

Recommendations

In the future I advise to reproduce some experimental work, with improved methodology, to reinforce our conclusions. Repeating of the PLA & PP dilution series test, followed up by the multiple stressor scenario, while using the same algae cultivation method would be recommended to ensure the cause of the differences in LDR's observed between the tests in this research. Furthermore, more elaborated tests of the next generation trial test is necessary to explain to what extent PLA leachates exhibit estrogenic activity and which other factors may influence the reproduction rates of *N. spinipes*.

Future implications

This research indicated that exposure to PLA leachates harms the survival of the brackish water copepod *N. spinipes* and that the vulnerability of the organism increased under a multiple stressor scenario. Therefore, this research can be used as an incentive for more strict regulations regarding plastic recycling and climate mitigation. Furthermore, this research provides a baseline for further testing of next generation effects under a multiple stressor scenario.

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Appendix 1: Multiwell plates

Plate scheme:

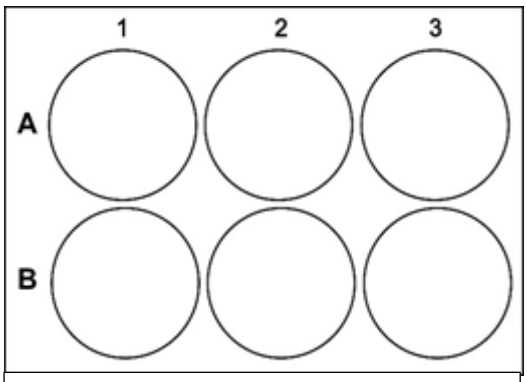

Figure 24. *Multiwell plates schematic*

Figure 25. *Multiwell plates picture*

Appendix 2 : Experimental design

Experiment 1:

This experiment was divided into two parts. The first experiment was to examine the (possible) toxicity of PLA leachates and the second to examine the (possible) toxicity of PP leachates. In both experiments there are 4 treatments (100%, 60%, 20% & 0% PLA or PP leachates). Each treatment had 8 replicates.

4 * 8 = 32

The multiwell plates have 6 cells per plate, meaning we needed 6 plates in total (Figure 26).

No. 6-well plate	No. Well/plate	Cumulative no. Wells
1	6	6
2	6	12
3	6	18
4	6	24
5	6	30
6	6	36
7	6	42
8	6	48
9	6	54
10	6	60

Figure 26. *Multiwell plates experiment 1*

The samples were labeled according to figure 27 (sample code). The treatment and replicate number are notated as well. Each cell contained +/-10 *nauplii*.

No.	Sample Code	Treatment	Replicates no. (wells)	Nauplii (per well)	Randomized no. (1-36)
1	EV_01.1_01	PLA / PP- 100%	1	10	6
2	EV_01.1_02	PLA / PP- 100%	2	10	8
3	EV_01.1_03	PLA / PP- 100%	3	10	36
4	EV_01.1_04	PLA / PP- 100%	4	10	14
5	EV_01.1_05	PLA / PP- 100%	5	10	20
6	EV_01.1_06	PLA / PP- 100%	6	10	35
7	EV_01.1_07	PLA / PP- 100%	7	10	4
8	EV_01.1_08	PLA / PP- 100%	8	10	9
9	EV_01.1_09	PLA / PP- 60%	1	10	27
10	EV_01.1_10	PLA / PP- 60%	2	10	5
11	EV_01.1_11	PLA / PP- 60%	3	10	7
12	EV_01.1_12	PLA / PP- 60%	4	10	33
13	EV_01.1_13	PLA / PP- 60%	5	10	19
14	EV_01.1_14	PLA / PP- 60%	6	10	26
15	EV_01.1_15	PLA / PP- 60%	7	10	34
16	EV_01.1_16	PLA / PP- 60%	8	10	12
17	EV_01.1_17	PLA / PP- 20%	1	10	30
18	EV_01.1_18	PLA / PP- 20%	2	10	10
19	EV_01.1_19	PLA / PP- 20%	3	10	1
20	EV_01.1_20	PLA / PP- 20%	4	10	24
21	EV_01.1_21	PLA / PP- 20%	5	10	16
22	EV_01.1_22	PLA / PP- 20%	6	10	18
23	EV_01.1_23	PLA / PP- 20%	7	10	2
24	EV_01.1_24	PLA / PP- 20%	8	10	21
25	EV_01.1_25	Control (0%)	1	10	31
26	EV_01.1_26	Control (0%)	2	10	3
27	EV_01.1_27	Control (0%)	3	10	29
28	EV_01.1_28	Control (0%)	4	10	13
29	EV_01.1_29	Control (0%)	5	10	11
30	EV_01.1_30	Control (0%)	6	10	22
31	EV_01.1_31	Control (0%)	7	10	25
32	EV_01.1_32	Control (0%)	8	10	15
					32
		Total number of organisms		320	17
					28
					23

Figure 27. *Sample coding Experiment 1*

The samples were designated to a cell in a multiwell plate by using a random number generator (NumberGenerator, n.d.). This led to the experimental set up (Figure 28).

Experimental set up

	Plate 1				Plate 2				Plate 3		
	1	2	3		1	2	3		1	2	3
A	EV_01.1_19	EV_01.1_23	EV_01.1_26	A	EV_01.1_11	EV_01.1_02	EV_01.1_08	A	EV_01.1_28	EV_01.1_04	EV_01.1_32
B	EV_01.1_07	EV_01.1_10	EV_01.1_01	B	EV_01.1_18	EV_01.1_29	EV_01.1_16	B	EV_01.1_21	x	EV_01.1_22

	Plate 4				Plate 5				Plate 6		
	1	2	3		1	2	3		1	2	3
A	EV_01.1_13	EV_01.1_05	EV_01.1_24	A	EV_01.1_31	EV_01.1_14	EV_01.1_09	A	EV_01.1_25	x	EV_01.1_12
B	EV_01.1_30	x	EV_01.1_20	B	x	EV_01.1_27	EV_01.1_17	B	EV_01.1_15	EV_01.1_06	EV_01.1_03

Figure 28. *Experimental set up multiwell plates Experiment 1*

Figure 29 shows an explanatory image of the copepod larvae development test with PLA/PP leachates:

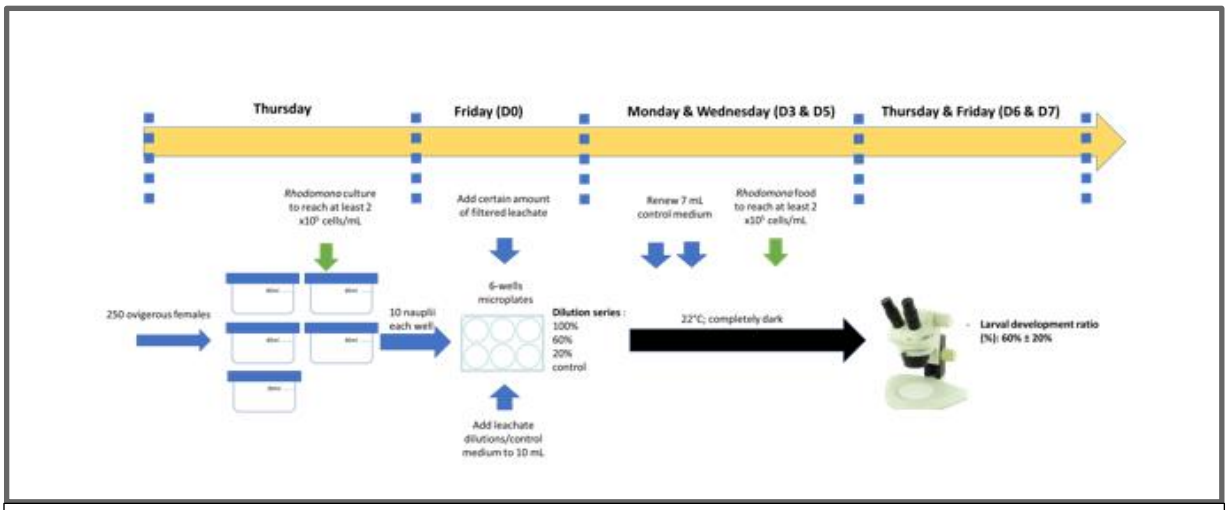

Figure 29 *.copepod larvae development test: Blind test with leachate from PLA,* (Niu,2021))

Experiment 2:

This experiment consisted of 6 treatments:

1) T0C0: 22 °C & control media

2) T0C1: 22 °C & 60% PLA leachates

3) T0C2: 22 °C & 60% PP leachates

4) T1C0 : 24 °C & control media

5) T1C1: 24 °C & 60% PLA leachates

6)T1C2: 24 °C & 60% PP leachates

Each treatment had 8 replicates. The treatments were split up in 2 groups: Group 1: T0 = treatment 1, 2 & 3 and Group 2: T1 = treatment 4,5 & 6. There were two separate groups, because the groups needed to be incubated at different temperatures.

3 * 8 = 24 per group = 4 multiwell plates per group (Figure 30).

	No. 6-well plate	No. Well/plate	Cumulative no. Wells
	1	6	6
	2	6	12
	3	6	18
2x	4	6	24
	5	6	30
	6	6	36
	7	6	42
	8	6	48
	9	6	54
	10	6	60

Figure 30. *Multiwell plates experiment 2*

The samples were labeled according to Figure 31 (sample code). The treatment and replicate number are notated as well. Each cell contained +/-10 *nauplii*.

No.	Sample Code	Temperature	Leachates Treatment	Replicates no. (wells)	Nauplii (per well)	Randomized no. (1-24)
1	EV_02_01	Control	Control	1	10	11
2	EV_02_02	Control	Control	2	10	16
3	EV_02_03	Control	Control	3	10	1
4	EV_02_04	Control	Control	4	10	22
5	EV_02_05	Control	Control	5	10	24
6	EV_02_06	Control	Control	6	10	5
7	EV_02_07	Control	Control	7	10	21
8	EV_02_08	Control	Control	8	10	19
9	EV_02_09	Control	PLA	1	10	10
10	EV_02_10	Control	PLA	2	10	8
11	EV_02_11	Control	PLA	3	10	20
12	EV_02_12	Control	PLA	4	10	2
13	EV_02_13	Control	PLA	5	10	15
14	EV_02_14	Control	PLA	6	10	17
15	EV_02_15	Control	PLA	7	10	12
16	EV_02_16	Control	PLA	8	10	7
17	EV_02_17	Control	PP	1	10	6
18	EV_02_18	Control	PP	2	10	23
19	EV_02_19	Control	PP	3	10	4
20	EV 02 20	Control	PP	4	10	9
21	EV_02_21	Control	PP	5	10	13
22	EV_02_22	Control	PP	6	10	18
23	EV_02_23	Control	PP	7	10	3
24	EV_02_24	Control	PP	8	10	14
17	EV_02_17	"+2C" - 3	Control	1	10	2
18	EV_02_18	"+2C" - 3	Control	2	10	16
19	EV_02_19	"+2C" - 3	Control	3	10	24
20	EV_02_20	"+2C" - 3	Control	4	10	13
21	EV_02_21	"+2C" - 3	Control	5	10	8
22	EV_02_22	"+2C" - 3	Control	6	10	19
23	EV_02_23	"+2C" - 3	Control	7	10	10
24	EV_02_24	"+2C" - 3	Control	8	10	22
25	EV_02_25	"+2C" - 1	PLA	1	10	21
26	EV_02_26	"+2C" - 1	PLA	2	10	14
27	EV_02_27	"+2C" - 1	PLA	3	10	15
28	EV_02_28	"+2C" - 1	PLA	4	10	7
29	EV_02_29	"+2C" - 1	PLA	5	10	20
30	EV_02_30	"+2C" - 1	PLA	6	10	1
31	EV_02_31	"+2C" - 1	PLA	7	10	18
32	EV_02_32	"+2C" - 1	PLA	8	10	3
33	EV_02_33	"+2C" - 1	PP	1	10	11
34	EV_02_34	"+2C" - 1	PP	2	10	9
35	EV_02_35	"+2C" - 1	PP	3	10	12
36	EV_02_36	"+2C" - 1	PP	4	10	23
37	EV_02_37	"+2C" - 1	PP	5	10	6
38	EV_02_38	"+2C" - 1	PP	6	10	17
39	EV_02_39	"+2C" - 1	PP	7	10	4
40	EV_02_40	"+2C" - 1	PP	8	10	5
			Total number of organisms		**480**	

Figure 31 .*Sample coding Experiment 2*

The samples were designated to a cell in a multiwell plate by using a random number generator (NumberGenerator, n.d.). This led to the experimental set up (Figure 32).

Experimental setup

Control		Plate 1			Plate 2			Plate 3			Plate 4		
		1	2	3	1	2	3	1	2	3	1	2	3
	A	EV_02_03	EV_02_12	EV_02_23	EV_02_16	EV_02_10	EV_02_20	EV_02_21	EV_02_24	EV_02_13	EV_02_08	EV_02_11	EV_02_07
	B	EV_02_19	EV_02_06	EV_02_17	EV_02_09	EV_02_01	EV_02_15	EV_02_02	EV_02_14	EV_02_22	EV_02_04	EV_02_18	EV_02_05
"+2C"		Plate 1			Plate 2			Plate 3			Plate 4		
		1	2	3	1	2	3	1	2	3	1	2	3
	A	EV_02_30	EV_02_17	EV_02_32	EV_02_28	EV_02_21	EV_02_34	EV_02_20	EV_02_26	EV_02_27	EV_02_22	EV_02_29	EV_02_21
	B	EV_02_39	EV_02_40	EV_02_37	EV_02_23	EV_02_11	EV_02_35	EV_02_18	EV_02_38	EV_02_31	EV_02_24	EV_02_36	EV_02_19

Figure 32. *Experimental set up multiwell plates Experiment 2*

Figure 33 shows an explanatory image of the multiple stressor test:

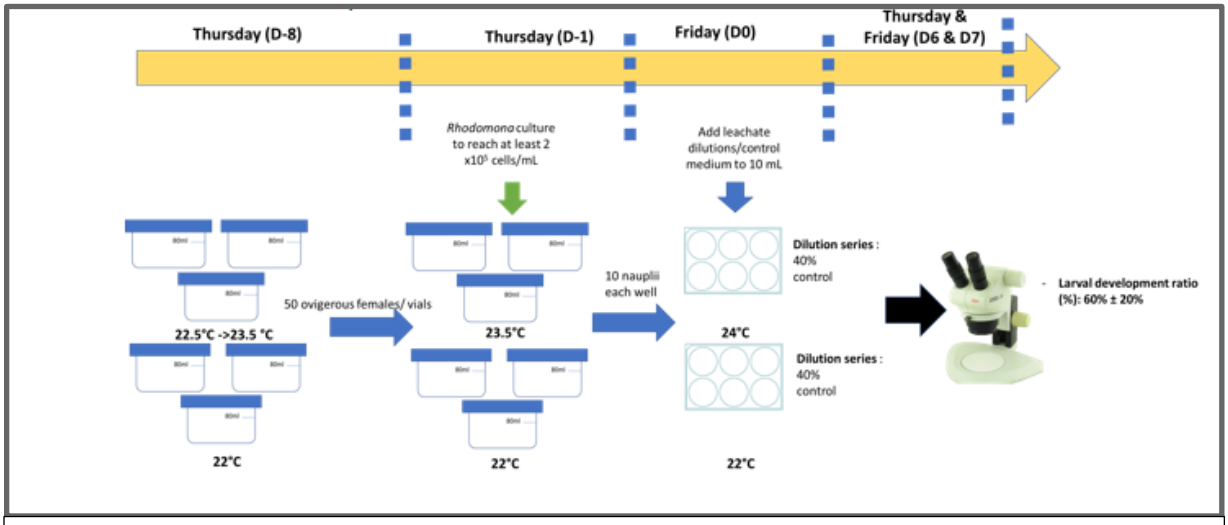

Figure 33. *Ecotoxicity: copepod larvae development test multiple-stressor test with leachate from PLA and raised temperature,*(Niu,2021).

Experiment 3:

In this experiment we used the survivors: copepods*/nauplii* (depended on the LDR after the test) from experiment 2. This experiment was a follow up on the multiple stressor trial. In this test we used a 40% leachate concentration based on the dilution series test. Therefore this experiment consisted of the same 4 treatments, as experiment 2, but at a lower leachate concentration.

1) T0C0: 22 °C & control media

2) T0C1: 22 °C & 40% PLA leachates

3) T1C0 : 24 °C & control media

4) T1C1: 24 °C & 40% PLA leachates

Each treatment had 4 replicates. The treatments were split up in 2 groups: Group 1: T0 = treatment 1 & 2 and Group 2:T1 = treatment 3 & 4. There are two separate groups, because the groups needed to be incubated at different temperatures.

2 * 4 = 8 per group = 2 multiwell plates per group (Figure 34).

	No. 6-well plate	No. Well/plate	Cumulative no. Wells
	1	6	6
2x	2	6	12
	3	6	18
	4	6	24
	5	6	30
	6	6	36
	7	6	42
	8	6	48
	9	6	54
	10	6	60
	11	6	66

Figure 34. *Multiwell plates Experiment 3*

The samples were labeled according to Figure 35 (sample code). The treatment and replicate number were notated as well. Each cell contained 20 adults.

No.	Sample Code	Temperature	Leachates Treatmer	Replicates no. (wells)	Nauplii (per well)	Randomized no. (1-12)
1	EV_03_01	Control	Control	1	20	5
2	EV_03_02	Control	Control	2	20	3
3	EV_03_03	Control	Control	3	20	11
4	EV_03_04	Control	Control	4	20	9
5	EV_03_05	Control	PLA	1	20	8
6	EV_03_06	Control	PLA	2	20	1
7	EV_03_07	Control	PLA	3	20	4
8	EV_03_08	Control	PLA	4	20	10
9	EV_03_09	"+2C" - 3	Control	1	20	1
10	EV_03_10	"+2C" - 3	Control	2	20	9
11	EV_03_11	"+2C" - 3	Control	3	20	7
12	EV_03_12	"+2C" - 3	Control	4	20	3
13	EV_03_13	"+2C" - 1	PLA	1	20	10
14	EV_03_14	"+2C" - 1	PLA	2	20	6
15	EV_03_15	"+2C" - 1	PLA	3	20	2
16	EV_03_16	"+2C" - 1	PLA	4	20	8
					320	Total number of organisms

Figure 35 .*Sample coding Experiment 3*

The samples were designated to a cell in a multiwell plate by using a random number generator (NumberGenerator, n.d.). This led to the experimental set up (Figure 36).

Experimental set up

Control

		Plate 1				Plate 2		
		1	2	3		1	2	3
	A	EV_03_06	x	EV_03_02	A	x	EV_03_05	EV_03_04
	B	Ev_03_07	EV_03_01	x	B	EV_03_08	EV_03_03	x

"+2C"

		Plate 1				Plate 2		
		1	2	3		1	2	3
	A	EV_03_09	EV_03_15	EV_03_12	A	EV_03_11	EV_03_16	EV_03_10
	B	x	x	EV_03_14	B	EV_03_13	x	x

Figure 36 .*Experimental set up multiwell plates Experiment 3*

Figure 37 shows an extra explanatory image of the trial experiment.

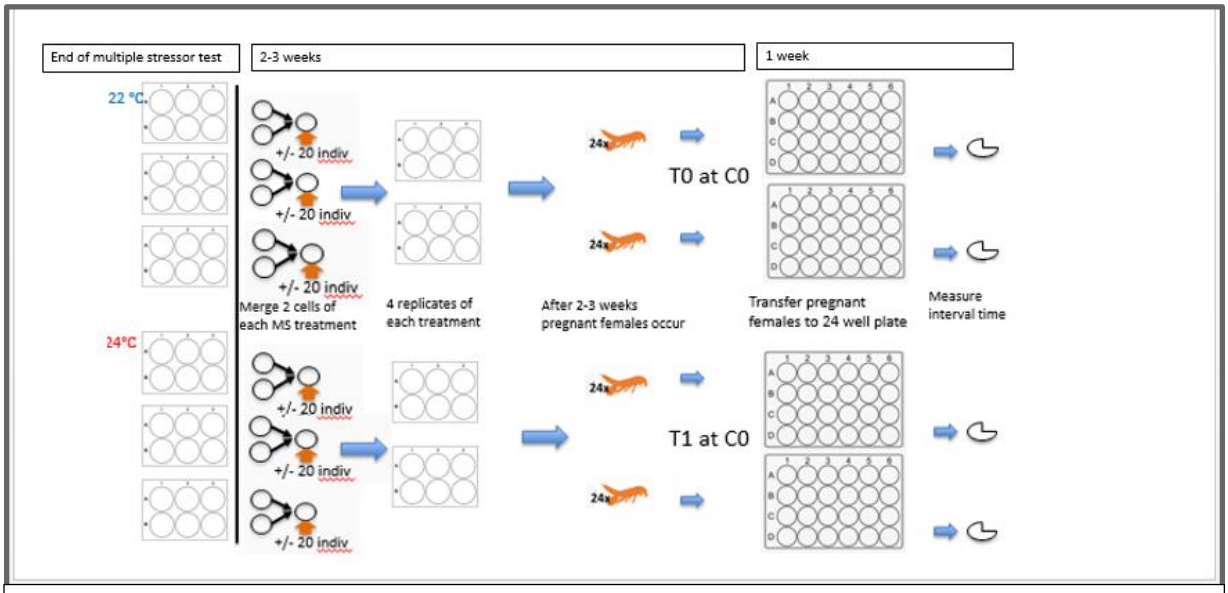

Figure 37. *Shematic design Next generation trial test*

Appendix 3: Material list

This material list is provided by ISO (2016)

Test organism

- Harpacticoid copepod *Nitokra spinipes* (culture)

Algae (copepods feeding):

- *Rhodomonas salina* (culture)

Reagents:

- Natural seawater
- NaOH (Supplier)
- 33% HCl (Supplier)
- 70% Ethanol (Chem-lab NV)
- Decon 90 (REF, SUPPLIER)
- Self-reinforced Polylactic acid (SR-PLA) sheet (thickness = 1.33 mm, supplied by Centexbel)
- Self-reinforced Polypropylene (SR-PP) sheet (thickness = 1.33 mm, supplied by Centexbel)

Equipment:

- Incubator (HERATHERM, Thermo Scientific)
- Laminar Flow Cabinet (HERAGUARD, Thermo Scientific)
- Low-magnifying stereo microscope (Lecia)
- Inverted microscope (Lecia)
- Neubauer improved counting chamber with cover slides (BLAUBRAND®) – 1 chamber
- Oven (BINDER)
- pH-meter (LLG)
- Temperature meter (S/N D194883,VWR)
- Fridge
- Hotplate (VWR)
- Balance (VWR)
- Magnetic stirrers
- Digital seawater refractometer (HANNA)
- Filtration Set (inlet / outlet tubing, 0.2 µm filter, the pump and tubing holder)
- Bunsen burner

Glassware & Other Consumables:

- Crystallization dishes with plastic lid, maximum volume
- 150 mL – 3 dishes
- Polystyrene 6-well microplate – 7 plates
- Glass Petri dishes (60mm) - 12 dishes
- Glass amber bottles with plastic cap 1000 mL
- Beakers 250 mL
- Wide pipette
- Lab coat, nitrile protection gloves and lab goggles

- Clean lint-free tissue paper
- Metal bucket (5 L) to wash glassware
- 0.22 µm air filters – 27
- Parafilm
- Steel scissor
- Teflon tape
- Red/black markers
- Aluminium foils

Appendix 4: Method

The experiments described below were conducted by following the toxicity blind test protocol of ISO (International Organization for Standardization)(2016) and modified versions of this protocol specifically designed for the pioneer testing of multiple stressor and next generation effects. The ISO protocol (2016) is provided by VLIZ , due to the confidential rights of this document the method will be described broadly.

Prior to the multiple stressor test, a dilution series test with PLA and PP leachates was conducted. This was done to test if there was any significant effect caused by the exposure to leachates of bio and/or synthetic-based plastics on the survival and development of *N. spinipes* and if so, to determine the EC50 of this particular leachate. The EC50 was used for the main test of this research: the multiple stressor test.

Protocol dilution series blind test

1. Quality control

Prior to the start of the experiment all glassware used is washed with Decon 90 solution and Milli-Q water and continuously sterilized by heating till 170 °C for a 2 hour period. The workplace, a flow cabinet is treated with a 70% ethanol solution.

2. Preparation of the control medium

500 mL seawater is diluted in a 1:3.5 ratio with deionized water. The seawater must have a pH of 7PSU if this is not the case this must be artificially altered. The diluted seawater is filtered with a 2 micrometer sieve to extract any plastic particles present, leaving a pure leachate concentration. The pH and salinity of the sieved water is measured. The pH of control media must stay within 6 and 9 at all times. The medium should be placed in a dark spot at 4 °C. The control media is conditioned hours prior to use.

3. Preparation of leachates

3 weeks prior to the test:

Preparation of leachates for PLA and PP is conducted separately. The plastic flakes are divided evenly in 3 groups. Weighing the flakes must lead to an equal weight for each group. In three sterilized milk bottles the flakes are prepared together with 200 mL of control medium. The bottle is capped with Teflon tape and additionally covered with parafilm. The bottles are wrapped in aluminum foil and placed on the orbital shaker at 22 °C for 21 days.

4. Preparation of algae suspension

1 week prior to use:

The flowcabinet is sterilized with 70% ethanol and the executor of the experiment works with gloves and a lab coat in the sterilized environment. 200 mL of sterilized L1 medium is added together with 20 mL of R. salina culture in each 500 mL Erlenmeyer flask (2 flasks in total). Continuously the flasks are sealed with a sterilized cotton plug and labeled accordingly to the project. The algae suspension flasks are then placed in the climate room.

Prior to use:

4 centrifuge tubes are prepared and labeled according to the dilution series. In the sterilized work environment of the flowcabinet 10 mL of algae culture is poured into each centrifuge tube and centrifuged at 1000 rpm for a 5 minute period. The fluid in the centrifuge tubes is continously discharged and filled up again with 10 mL of the corresponding media or dilution. All tubes are shaken prior to use to homogenize the algae concentration in the tube. To each cell in the multiwell plates 1 mL of the final product is added.

5. Production of *nauplii* to be used in the test

The health of the Nicotra is monitored for 3 weeks in advance of the test by microscope. 1 day prior to the test 250 ovigerous females are taken with a pipette and randomly and equally divided over 5 crystallization dishes. To each dish 1 knife tip of mashed salmon (the food) is added. The cultures are incubated in the dark at 22 °C.

6. Preparation of leachates dilution series

The bottles containing the leachates are taken off the shaker and filtered through a 0.22 micrometer filter into a sterilized amber glass bottle, this bottle is labeled as the leachate stock. Prior to the making of the dilutions, the pH and salinity of the leachate stock are checked. The leachate is transferred into 4 sterilized 250 mL amber glass bottles, labelled as: C0, C1, C2, & C3. The flasks contain the following dilutions:

C0: 250 mL control medium (0%)

C1: 50 mL leachates + 200 mL control medium (20%)

C2: 150 mL leachates + 100 mL control medium (60%)

C3: 250 mL leachates (100%)

All flasks are conditioned 24hours prior to use.

7. Set-up

6 clean 6-well microplates are labelled and marked with a 3 mL line in red and a 10 mL line in black on the microplates. Continuously 5 mL of each prepared dilution is transferred into the 6-well microplates. The dilutions are divided randomly over the 6-well microplates by using a randomizer computer program. +/- 10 *nauplii* are transferred with a pipette into the dishes and the exact number of *nauplii* in each concentration is noted down in the data sheet. Each dish is filled up further till the black mark with its according dilution. Additionally, 1 mL of algae suspension is added to each dish. Also 1 separate multiwell plate, labeled as “extra” is prepared. In this plate 3 random cells are filled with control medium. Those cells will be used for measuring the validity parameters during the test. All microplates are then covered with parafilm and their lid, to avoid evaporation.

8. Incubation

The microplates are incubated for 6-7 days at 22 °C in the dark.

9. Maintenance

At day 3 & 5 the microplates are taken from the incubator. On these days 7 mL of control media / dilution concentration is taken out of the cells with a 10 mL pipet and transferred into an empty multiwell plate, labeled similar to the original plate. This plate is checked for the presence of *nauplii* or copepods that might be transferred with the fluid by accident. If this is the case the organisms are transferred back to the cell they came out with a 10 microliter pipet. This is done for each cell in each plate. If the fluid is clear (no organisms present) it is discharged into the sink and 6 mL fresh media /

dilution is added to each cell in the multiwell plates. All cells receive 1 mL of algae feed on the days of maintenance as well. For the cells in the "extra" plate 7 mL is taken out and renewed with 7 mL fresh control media as well. Before and after renewing of the extra cells the pH & salinity is measured to check the validity criteria. At day 5,6 & 7 the number of *nauplii* and copepodites in all wells and control dishes are counted, with use of a microscope

(ISO, 2016), (R Core Team, 2019) & (VLIZ, 2021)

10. Data processing

The data of this test was analyzed by performing a Kruskal-Wallis test. This test was used as the data did not meet the assumptions of ANOVA. The results of the leachate dilution test with PLA and the test with PP led to the decision on which type(s) of plastic the multiple stressor test was executed with. Accordingly the test concentration of plastic leachates in the multiple stressor test was selected based on the EC50. The EC50 is a statistical parameter that indicates the concentration of a toxicant that results in a certain effect (e.g. delay in development) in 50% of the tested population compared to the control group. The EC50 is calculated by identifying the halfway response between the baseline and maximum response(GraphPad Software, LLC, n.d.).

The *EC10 value is consequently the toxicity concentration applied to a test group that results in a 10% effect (e.g. delay in development) compared to the control group. The EC10 value is used in this report to compare studies. The EC10 value is calculated with the formula:

$EC_F=(F/(100-F)^{1/H} * EC50$

In which:

EC_F= EC (any percentage)

F= any percentage

H= hillslope

EC50= previously obtained EC50 from the same dataset

Validity Criteria
The tests will be considered valid according to the ISO protocol (ISO,2016) if the following requirements are met:

- The pH must remain within 6 and 9 during all tests
- The conductivity should not vary more than 10% compared to the control start value in all tests
- The average control copepodites fraction should be 60 % +/- 20% of surviving animals at the end of exposure
- The average mortality of animals in the control(s) on the day observation should stay within 20%

Protocol multiple stressor test

1. Quality control

Prior to the start of the experiment all glassware used is washed with Decon 90 solution and Milli-Q water and continuously sterilized by heating till 170 °C for a 2 hour period. The workplace, a flow cabinet is treated with a 70% ethanol solution.

2. Preparation of the control medium

500 mL seawater is diluted in a 1:3.5 ratio with deionized water. The seawater must have a salinity of 7PSU if this is not the case this is artificially altered. The diluted seawater is filtered with a 2 micrometer sieve to extract any plastic particles present, leaving a pure leachate concentration. The pH and salinity of the sieved water are measured. The pH of control media must stay within 6 and 9 at all times. The medium is placed in a dark spot at 4 °C. The control medium is conditioned hours prior to use.

3. Preparation of leachates

3 weeks prior to the test:

Based on the previous executed dilution series blind test, PLA and PP are both selected for further testing purposes. Therefore leachate of both treatments are prepared. 3 times +/- 16 grams of PLA (this is equal to 3 times +/-7 flakes of 2 by 7.5 cm PLA) are divided evenly over three sterilized milk bottles. The same is done for the PP flakes. To each bottle 200 mL of control medium is added. The bottle is capped with Teflon tape and additionally covered with parafilm. The bottles are wrapped in aluminum foil and placed on the orbital shaker at 22 °C for 21 days at 80 rpm.

4. Preparation of algae suspension

1 week prior to use:

The flow cabinet is sterilized with 70% ethanol and the executor of the experiment works with gloves and a lab coat in the sterilized environment. 200 mL of sterilized L1 medium is added together with 20 mL of R. salina culture in each 500 mL Erlenmeyer flask (2 flasks in total). Continuously the flasks are sealed with a sterilized cotton plug and labeled accordingly to the project. The algae suspension flasks are then placed in the climate room.

Prior to use:

4 centrifuge tubes are prepared and labeled according to the treatment (temperature and concentration). In the sterilized work environment of the flow cabinet 10 mL of algae culture is transferred with a 10 mL pipet into each centrifuge tube and centrifuged at 1000 rpm for a 5 minute period. The fluid in the centrifuge tubes is discharged and filled up again with 10 mL of the corresponding treatment. All tubes are shaken prior to use to homogenize the algae concentration. For feeding 1 mL of the corresponding algae + treatment mixture is added to each cell.

5. Acclimatization & Production of *nauplii* to be used in the test

2 weeks in advance to the test 12 cultures of *N. spinipes* are split up over 2 incubators. One maintains a constant temperature of 22 °C. The second incubator with the other 6 cultures starts at 22 °C as

well, but is increased every third/fourth day with 0.5 °C, till it reaches a temperature of 24 °C on the day prior to the test. This is the maximum estimated temperature rise according to IPCC (2019), *N. spinipes* potentially has to deal with by 2100. During those 2 weeks the health of the Nicotra is monitored by microscope. 1 day prior to the test 3 precultures of 50 ovigerous females are taken with a pipette from the 6 cultures incubated at 22 °C and 3 precultures of 50 ovigerous females are taken from the 24 °C incubator. To each dish 1 knife tip of mashed salmon (the food) is added. The cultures are labeled according to their condition and placed back in the corresponding incubator.

6. Preparation of the leachate dilution

The bottles containing the leachates are taken off the shaker and filtered through a 0.22 micrometer filter into a sterilized amber glass bottle, this bottle is labeled as the leachate stock. Prior to the making of the dilution, the pH and salinity of the leachate stock are checked. For the leachate dilution concentration used in this test a 60% PLA and PP dilution is selected. This is the EC50 concentration according to the corrected value of the PLA dilution series by the multiple stressor trial test. Four 250 mL sterilized amber glass bottles are labelled as: T0C0, T0C1, T1C0, & T1C1. The flasks contain the following treatments:

T0C0= 250 mL control media at 22 °C
T0C1= 150 mL control media + 100 mL PLA leachate (60% dilution) at 22 °C
T0C2= 150 mL control media + 100 mL PP leachate (60% dilution) at 22°C
T1C0= 250 mL control media at 24 °C
T1C1= 150 mL control media + 100 mL PLA leachate (60% dilution) at 24 °C
T1C2=150 mL control media + 100 mL PLA leachate (60% dilution) at 24 °C

9. Set-up

8 clean 6-well microplates are divided in two groups, the 2 sets of 4 plates are labeled using a randomizer. First the 4 plates of the T0 treatments are filled with 5 mL of the assigned concentration. To each cell +/- 10 *nauplii* of the precultures from the 22 °C incubator are added randomly. The exact number of *nauplii* in each cell is written down on the data sheet. Continuously 4 mL more of the assigned concentration is added into the cells of the 6-well microplates. 1mL of algae food (see step 4) is added to the cells as well, after which they are placed back in the incubator at 22 °C. For the second group of T1 the same is done. From the 3 precultures incubated at 24 °C +/- 10 *nauplii* are randomly transferred to each cell. After which again the exact number is noted down in the datasheet and 4 more mL of the assigned treatment is added to the cells as well as the algae food. The T1 multiwell plates are incubated at 24 °C again. For T0 & T1 both a multiwell plate labeled as “extra” is prepared. To this plate 10 mL control media is transferred. The pH, salinity and temperature of the control media in the extra plates is measured and written down. The plate of T0 is continuously placed in the 22 °C incubator with the other multiwell plates and so is the T1 plate placed in the 24 °C incubator as well.

10. Incubation

The microplates are placed in the incubators for 6-7 days in the dark.

11. Maintenance

At day 3 & 5 the microplates are taken from the incubator. On these days 7 mL of control media / 60% dilution concentration is taken out of the cells with a 10 mL pipet and transferred into an empty multiwell plate, labeled similar to the original plate. This plate is checked for the presence of *nauplii* or copepods that might be transferred with the fluid by accident. If this is the case the organisms are transferred back to the cell they came out with a 10 microliter pipet. This is done for each cell in each plate. If the fluid is clear (no organisms present) it is discharged into the sink and 6 mL fresh media / 60% dilution is added to each cell in the multiwell plates. All cells receive 1 mL of algae feed on the days of maintenance as well. For the cells in the "extra" plate 7 mL is taken out and renewed with 7 mL fresh control media as well. Before and after renewing of the extra cells the pH & salinity is measured to check the validity criteria. At day 5,6 & 7 the number of *nauplii* and copepodites in all wells and control dishes are counted, with use of a microscope.

Validity Criteria
The tests will be considered valid if the following requirements are met (ISO,2016):

- The pH must remain within 6 and 9 during all tests
- The conductivity should not vary more than 10% compared to the control start value in all tests
- The average control copepodites fraction should be 60 % +/- 20% of surviving animals at the end of exposure
- The average mortality of animals in the control(s) on the day observation should stay within 20%

12. Data processing

The data of this test was analyzed with an ANOVA test. The data fit the assumptions of normality and homogeneity (tested with Shapiro-Wilk normality test and Levene's test test for homogeneity of varience). The results of this test led to the decision on which type of plastic was used for further testing in the next generation test.

Protocol next generation test

This test can be performed directly after the multiple stressor test described above or performed completely separate. The first protocol is for execution directly after the multiple stressor test. This the protocol followed in this experiment. As it was performed directly after the multiple stressor trial test the leachate concentration used in this test was 40%. Based on this test the leachate concentration in the full next generation protocol is adapted to 60%.

Note: For this experiment 2 times the normal volume of leachates is necessary (see full protocol next generation test for instructions).

1.Set up

4 clean 6-well microplates are divided into two groups, the 2 sets of 2 plates are labeled using a randomizer. This must be done in groups to avoid mixing of the temperatures within the test plates. First the 2 plates of the T0 treatments are filled with 5 mL of the assigned concentration (C0 or C1). To each cell +/- 20 adults/ *nauplii* (depending on the LDR of the multiple stressor test) are added

from the previously performed multiple stressor test. This is done by combining 2 replicates of the same treatment for each treatment and transferring them to the new test cell of the next generation experiment multiwell-plates. The exact number of *nauplii*/copepods in each cell is written down on the data sheet. Continuously 4 more mL of the assigned concentration is added into the cells of the 6-well microplates. 1mL of algae food (see step 4) is added to the cells as well, after which they are placed back in the incubator at 22 °C. For the second group (the T1) the same is done. From the multiple stressor test 2 replicates of the same T1 treatments are randomly merged together and +/- 20 *nauplii*/copepods are transferred to each cell of the next generation experiment multiwell-plates. The exact number of organisms per cell is noted down in the datasheet and 4 more mL of the assigned treatment is added to the cells as well as the algae food. The T1 multiwell plates are incubated at 24 °C again. For T0 & T1 both a multiwell plate labeled as "extra" is prepared. To this plate 10 mL control media is transferred. The pH, salinity and temperature of the control media in the extra plates must be measured and written down. The extra plate of T0 is continuously placed in the 22 °C incubator with the other multiwell plates and so is the extra plate of T1 placed in the 24 °C incubator as well.

2. Incubation

The microplates are placed in the incubators for 6-7 days in the dark.

3.Maintenance

(Day 2-14) On the 3rd, 5th and 7th day of the first week (as the experiment starts on the Friday previous to the maintenance weeks) and the 1st 3rd and 5th day of the second week, the microplates are taken from the incubator. On these days 7 mL of control media / 40% dilution concentration is taken out of the cells with a 10 mL pipet and transferred into an empty multiwell plate, labeled similar to the original plate. This plate is checked for the presence of *nauplii* or copepods that might be transferred with the fluid by accident. If this is the case the organisms are transferred back to the cell they came out with a 10 microliter pipet. This is done for each cell in each plate. If the fluid is clear (no organisms present) it is discharged into the sink and 6 mL fresh media / 60% dilution is added to each cell in the multiwell plates. All cells receive 1 mL of algae feed on the days of maintenance as well. For the cells in the "extra" plates 7 mL is taken out and renewed with 7 mL fresh control media as well. Before and after renewing of the extra cells the pH & salinity is measured to check the validity criteria.

Every day of this period the cells are checked on the presence of ovigerous females. When present the number of ovigerous females and the cell they came out of is written down. The ovigerous females are transferred into a separate 24-well plate. To this cell 1 mL of control media at 22 or 24 °C is added according to the treatment plus 100 micro liters of algae food. For each treatment a separate 24-well plate is prepared. From the point onwards that the first ovigerous female is transferred to the 24-well plate, this plate is monitored on a daily basis. For each ovigerous female individually the brood interval time and spawning success is tracked. When the females spawn, the *nauplii* are carefully transferred out of the cell to a separate 24-well plate. To this plate 1 mL control media at 22 or 24 °C is added and 1 mL formalin. There is one *nauplii* plate for each treatment.

(Day 14)
The 6-well plates containing the +/- 20 organisms are no longer maintained or checked on the presence of ovigerous females (it is the 21st day of their life cycle). The 24- well plates containing the

ovigerous females are still monitored on a daily basis to track the brood interval time and spawning success.

(Day 15-21)
The situation in the 24-well plates containing the ovigerous females is monitored for one more week. On the 1st, 3rd and 5th they are fed 100 microliter algae and the evaporation in the cells is corrected by adding control media. During this week the spawning success of the ovigerous females is measured based on the broodinterval time and the actual presence of *naupli*i. *Nauplii* spawned during this week are transferred to the separate *nauplii* plate.

Validity Criteria
The tests will be considered valid if the following requirements are met (ISO,2016):

- The pH must remain within 6 and 9 during all tests
- The conductivity should not vary more than 10% compared to the control start value in all tests
- The average control copepodites fraction should be 60 % +/- 20% of surviving animals at the end of exposure
- The average mortality of animals in the control(s) on the day observation should stay within 20%

4.Data processing

The data of this experiment was analyzed qualitative and preliminary.

Full protocol next generation test

1. Quality control

Prior to the start of the experiment all glassware used is washed with Decon 90 solution and Milli-Q water and continuously sterilized by heating till 170 °C for a 2 hour period. The workplace, a flow cabinet is treated with a 70% ethanol solution.

2. Preparation of the control medium

500 mL seawater is diluted in a 1:3.5 ratio with deionized water. The seawater must have a salinity of 7PSU if this is not the case this is artificially altered. The diluted seawater is filtered with a 2 micrometer sieve to extract any plastic particles present, leaving a pure leachate concentration. The pH and salinity of the sieved water are measured. The pH of control media must stay within 6 and 9 at all times. The medium is placed in a dark spot at 4 °C. The control medium is conditioned hours prior to use.

3. Preparation of leachates

3 weeks prior to the test:

Based on the previously executed multiple stressor test, PLA is selected for further testing purposes. Therefore PLA leachates are prepared. 3 times +/- 32 grams of PLA (this is equal to 3 times +/-14 flakes of 2 by 7.5 cm PLA) are divided evenly over three sterilized milk bottles. To each bottle 400 mL of control medium is added. The bottle is capped with Teflon tape and additionally covered with parafilm. The bottles are wrapped in aluminum foil and placed on the orbital shaker at 22 °C for 21 days at 80 rpm.

4. Preparation of algae suspension

1 week prior to use:

The flow cabinet is sterilized with 70% ethanol and the executor of the experiment works with gloves and a lab coat in the sterilized environment. 200 mL of sterilized L1 medium is added together with 20 mL of R. salina culture in each 500 mL Erlenmeyer flask (2 flasks in total). Continuously the flasks are sealed with a sterilized cotton plug and labeled accordingly to the project. The algae suspension flasks are then placed in the climate room.

Prior to use:

4 centrifuge tubes are prepared and labeled according to the treatment (temperature and concentration). In the sterilized work environment of the flowcabinet 10 mL of algae culture is transferred with a 10 mL pipet into each centrifuge tube and centrifuged at 1000 rpm for a 5 minute period. The fluid in the centrifuge tubes is discharged and filled up again with 10 mL of the corresponding treatment. All tubes are shaken prior to use to homogenize the algae concentration. For feeding 1 mL of the corresponding algae + treatment mixture is added to each cell.

5. Acclimatization & Production of *nauplii* to be used in the test

2 weeks in advance to the test 12 cultures of N. Spinpes are split up over 2 incubators. One maintains a constant temperature of 22 °C. The second incubator with the other 6 cultures starts at 22 °C as well, but is increased every third/fourth day with 0,5 °C, till it reaches a temperature of 24 °C on the day prior to the test. This is the maximum estimated temperature rise according to IPCC (2019), *N. spinipes* potentially has to deal with by 2100. During those 2 weeks the health of the Nicotra is monitored by microscope. 1 day prior to the test 3 precultures of 50 ovigerous females are taken with a pipette from the 6 cultures incubated at 22 °C and 3 precultures of 50 ovigerous females are taken from the 24 °C incubator. To each dish 1 knife tip of mashed salmon (the food) is added. The cultures are labeled according to their condition and placed back in the corresponding incubator.

6. Preparation of the leachate dilution

The bottles containing the leachates are taken off the shaker and filtered through a 0.22 micrometer filter into a sterilized amber glass bottle, this bottle is labeled as the leachate stock. Prior to the making of the dilution, the pH and salinity of the leachate stock is checked. For the leachate dilution concentration used in this test a 60% PLA dilution is selected. Four 250 mL sterilized amber glass bottles are labelled as: T0C0, T0C1, T1C0, & T1C1. The flasks contain the following treatments:

2x T0C0= 250 mL control media at 22 °C

2x T0C1= 150 mL control media + 100 mL PLA leachate (60% dilution) at 22 °C

2x T1C0= 250 mL control media at 24 °C

2x T1C1= 150 mL control media + 100 mL PLA leachate (60%dilution) at 24 °C

7.Set up

4 clean 6-well microplates are divided into two groups, the 2 sets of 2 plates are labeled using a randomizer. This is done in groups to avoid mixing of the temperatures within the test plates. First the 2 plates of the T0 treatments are filled with 5 mL of the assigned concentration (C0 or C1). To each cell +/- 20 *nauplii* from the precultures incubated at 22 °C incubator are added randomly. The exact number of *nauplii* in each cell is written down on the data sheet. Continuously 4 more mL of the assigned concentration is added into the cells of the 6-well microplates. 1mL of algae food (see step 4) is added to the cells as well, after which they are placed back in the incubator at 22 °C. For the second group of T1 the same is done. From the 3 precultures incubated at 24 °C +/- 20 *nauplii* are randomly transferred to each cell. After which again the exact number is noted down in the datasheet and 4 more mL of the assigned treatment is added to the cells as well as the algae food. For T0 & T1 both a multiwell plate labeled as "extra" is prepared. To this plate 10 mL control media is transferred. The pH, salinity and temperature of the control media in the extra plates are measured and written down. The extra plate of T0 is continuously placed in the 22 °C incubator with the other multiwell plates and so is the extra plate of T1 placed in the 24 °C incubator as well.

2. Incubation

The microplates are placed in the incubators for 6-7 days in the dark.

3.Maintenance

(Day 3-21) On the 3rd, 5th and 7th day of the first week (as the experiment starts on the Friday previous to the maintenance weeks) and the 1st 3rd and 5th day of the following weeks, the microplates are taken from the incubator. On these days 7 mL of control media / 60% dilution concentration is taken out of the cells with a 10 mL pipet and transferred into an empty multiwell plate, labeled similar to the original plate. This plate is checked for the presence of *nauplii* or copepods that might be transferred with the fluid by accident. If this is the case the organisms are transferred back to the cell they came out with a 10 microliter pipet. This is done for each cell in each plate. If the fluid is clear (no organisms present) it is discharged into the sink and 6 mL fresh media / 60% dilution is added to each cell in the multiwell plates. All cells receive 1 mL of algae feed on the days of maintenance as well. For the cells in the "extra" plates 7 mL is taken out and renewed with 7 mL fresh control media as well. Before and after renewing of the extra cells the pH & salinity is measured to check the validity criteria.

(Day 7) From this point onwards every day/ every second day of this period the cells are checked on the presence of ovigerous females. When present the number of ovigerous females and the cell the ovigerous female came out of is written down. The ovigerous female is transferred into a separate 24-well plate. To this cell 1 mL of control media at 22 or 24 °C is added according to the treatment plus 100 micro liters of algae food. For each treatment a separate 24-well plate is prepared. From the point onwards that the first ovigerous female is transferred to the 24-well plate, this plate is monitored on a daily basis. For each ovigerous female individually the brood interval time and spawning success are tracked. When the females spawn, the *nauplii* is carefully transferred out of the cell to a separate 24-well plate. To this plate 1 mL control media at 22 or 24 °C is added and 1 mL formalin. There is one *Nauplii* plate for each treatment.

(Day 21)

The 6-well plates containing the +/- 20 organisms are no longer maintained or checked on the presence of ovigerous females. The 24- well plates containing the ovigerous females are still monitored on a daily basis to track the brood interval time and spawning success.

(Day 21-28)
The situation in the 24-well plates containing the ovigerous females are monitored for one more week. On the 1st, 3rd and 5th they are fed 100 microliter algae and the evaporation in the cells is corrected by adding control media. During this week the spawning success of the ovigerous females is measured based on the brood interval time and the actual presence of *nauplii* in the cells. *Nauplii* spawned during this week are transferred to the separate *nauplii* plate..

Validity Criteria
The tests will be considered valid according to the ISO protocol (ISO,2016) if the following requirements are met:

- The pH must remain within 6 and 9 during all tests
- The conductivity should not vary more than 10% compared to the control start value in all tests
- The average control copepodites fraction should be 60 % +/- 20% of surviving animals at the end of exposure
- The average mortality of animals in the control(s) on the day observation should stay within 20%

Appendix 5: Planning

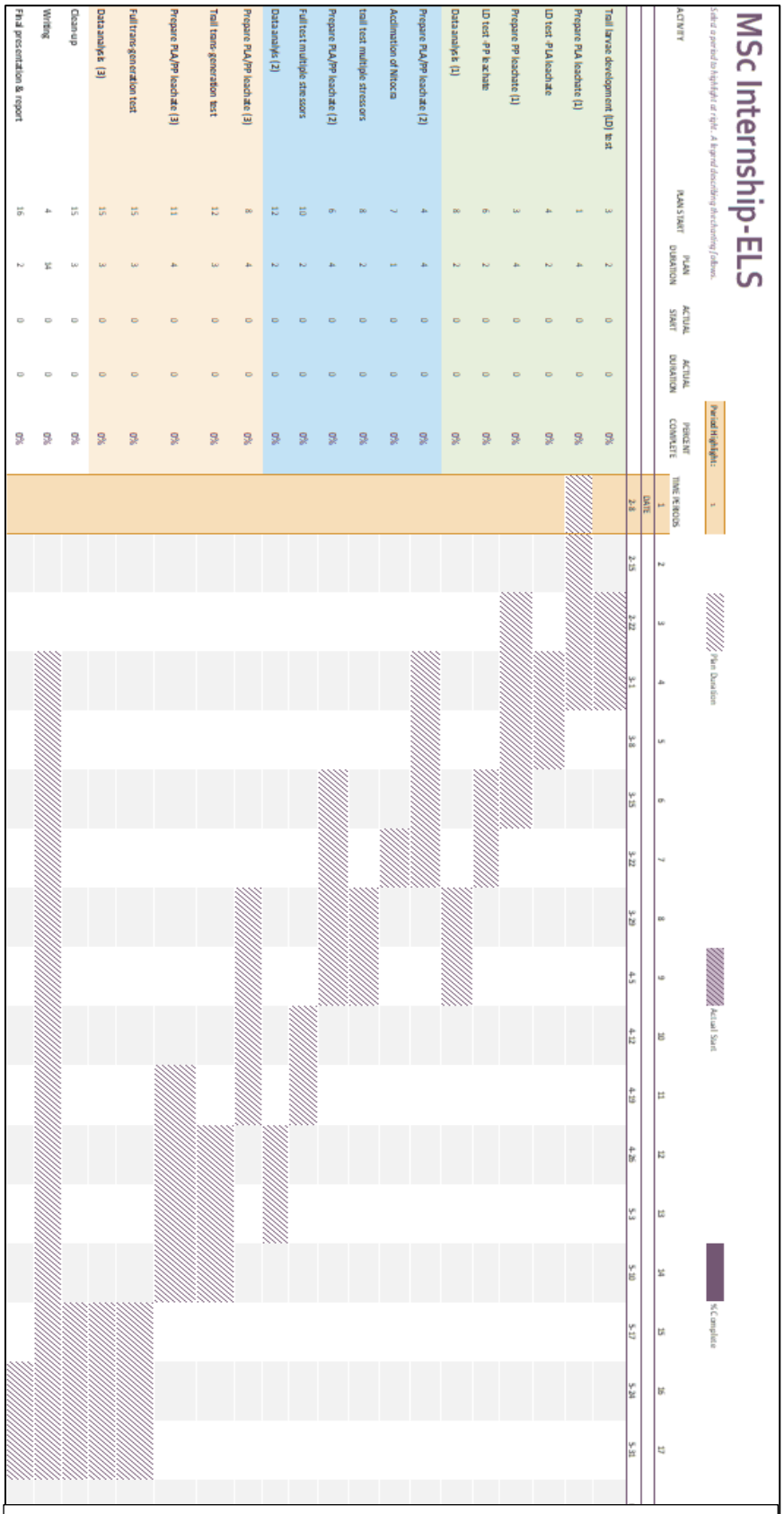

Figure 38 .*Gantt-chart planning internship period*

Appendix 6: Overview experiments

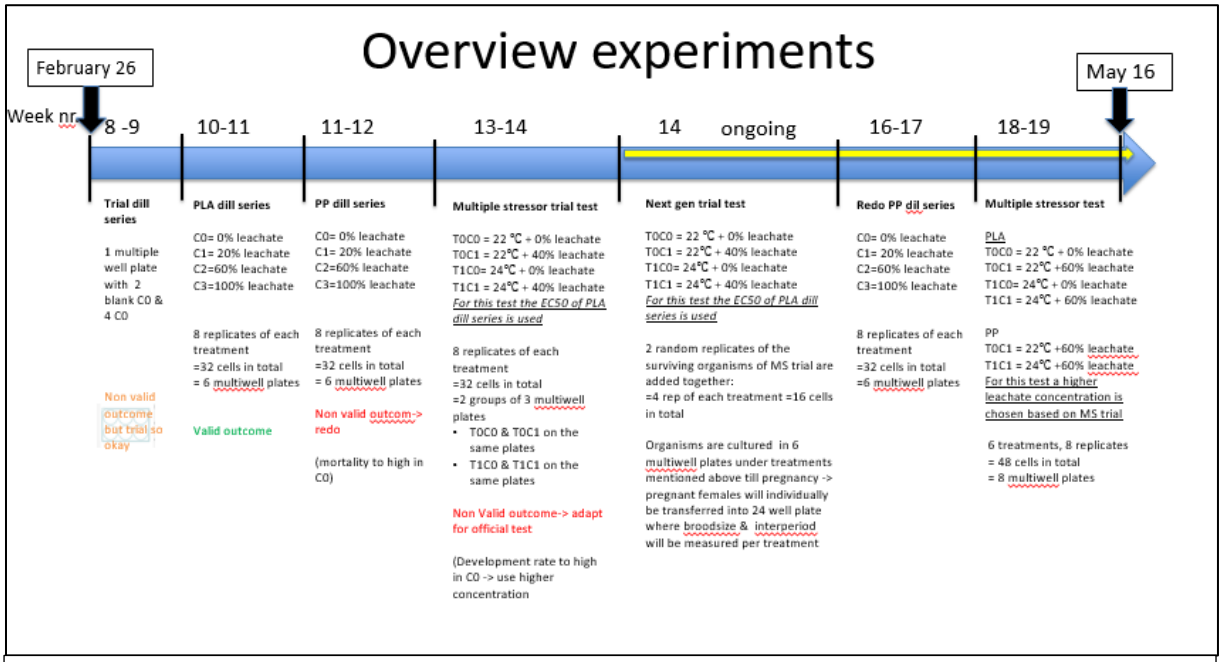

Figure 39 .*Overview experiments internship period*