FACULTY OF BIOSCIENCE ENGINEERING

EFFECTS OF PLASTIC POLLUTION AND OCEAN WARMING ON *PHAEODACTYLUM TRICORNUTUM* AND *NITOKRA SPINIPES*

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PREFACE

Completing this master's thesis marks the end of a meaningful academic journey, and I am excited to share the culmination of months of dedicated research, exploration, and introspection. As a passionate advocate for the ocean and its well-being, I am grateful to have worked on this topic.

Thank you to my promotor at Ghent University, Jana Asselman, for assigning this topic to me and for the support and guidance during this thesis journey. I would also like to thank my copromotor at VLIZ, Ana Catarino, for sharing her expert knowledge with me throughout the year. Her willingness to dedicate time to my project, even during busy periods, underscores her commitment to my academic growth. I am truly appreciative of her cheerful mentorship and the positive impact it has had on my research and overall experience. I would like to extend my sincere gratitude to my tutor, Zhiyue Niu, for being a constant source of guidance and support from the very beginning of this journey. His role in introducing me to the lab and providing ongoing assistance has been invaluable. I always felt I could reach out for help and receive timely advice, which I greatly appreciate.

Commuting to work in the lab in Ostend was not always easy (NMBS schedules considered), but I am grateful I got to know all the great people at VLIZ. I really enjoyed getting to know this company and being invited to the interesting seminars. A special thanks to my lab buddies, Rocío Soledad Pazos, Nathan Nault, and Lottie Linley for the nice conversations in-between experiments. I truly loved every moment spent by the seaside, and this connection to my passion for the ocean has added a special dimension to my thesis journey.

However, I always felt grateful to arrive back home as well. A sincere thank you to my family and partner, who showed me a great amount of love and encouragement throughout this academic endeavor. As well as to my two study buddies Zoé Bolluijt and Camine Vastiau, with whom I had many late-night study sessions, and shared moments of venting, which lightened the load and fostered a sense of unity and resilience.

In closing, I extend my heartfelt thanks to you, the reader. I hope the insights presented within these pages contribute meaningfully to our shared pursuit of knowledge and progress.

Yasmine De Witte

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SAMENVATTING

Menselijke activiteiten hebben geleid tot twee belangrijke milieustressoren: plasticvervuiling en klimaatverandering. Kunststoffen, bekend om hun gebruiksvriendelijkheid en veelzijdigheid, worden wereldwijd steeds meer geproduceerd. Hoge productiesnelheden, lage recyclingpercentages, het opstapelen van zwerfvuil en de persistentie van plastic materialen resulteren in ophopend, alomtegenwoordig plastic afval met verschillende groottecategorieën, waaronder microplastics (1 μm - 5 mm). Naast de fysieke aanwezigheid van microplastics, maakt het uitlogen van chemicaliën zoals monomeren en additieven uit kunststoffen in het milieu, bekend als *leachates* (Eng.), het probleem nog ingewikkelder. Zowel (micro)plastic deeltjes als leachates zijn zorgwekkend. Naarmate klimaatverandering vordert als gevolg van koolstofemissies door menselijke activiteiten, komt de opwarming van de oceanen naar voren als een bepalend gevolg hiervan. Deze tweede stressfactor kan mariene organismen op verschillende trofische niveaus beïnvloeden en kan de samenstelling van gemeenschappen veranderen.

Binnen deze context was het doel van deze studie om de effecten van plastic leachates op de groeisnelheid van algen te bepalen en om de gecombineerde effecten van leachates en de opwarming van de oceaan op de mobiliteit van roeipootkreeftjes te onderzoeken. Het onderzoek begon met het nagaan van de ecotoxiciteit van leachates op *Phaeodactylum tricornutum*, een diatomee die verantwoordelijk is voor de primaire productie in de oceaan. De resultaten toonden groeiremming aan, veroorzaakt door PVC leachates en leachates uit PLA dat zeewaterveroudering had ondergaan. Dit gaf nadruk op de invloed van materiaalverwering op de toxiciteit van leachates. Vervolgens werd de interactie tussen temperatuur en toxiciteit van plastic leachates onderzocht. Verrassend genoeg namen de toxische effecten van PVC leachates op volwassen *Nitokra spinipes*, een harpacticoïde roeipootkreeftje, niet significant toe bij een temperatuurstijging van 3 °C. Dit resultaat onderstreept de complexiteit van interacties tussen meerdere stressfactoren.

We concluderen dat deze uitgebreide studie inzichten verschaft over de complexe verbanden tussen plastic vervuiling, opwarming van de oceaan en aquatische organismen. Door de toxiciteit van plastic leachates en hun wisselwerking met temperatuurstress te onderzoeken, draagt het onderzoek bij aan het ontrafelen van mogelijke ecologische gevolgen. Uiteindelijk kan dit werk informatie bieden voor weloverwogen besluitvorming over duurzaam gebruik van plastic, rekening houdend met het beheer en behoud van mariene ecosystemen.

SUMMARY

Human activity has led to two major environmental stressors: plastic pollution and climate change. Plastics, renowned for their convenience and versatility, are being increasingly produced globally. High production rates, low recycling rates, extensive littering, and persistence of plastic materials result in accumulating, omnipresent plastic debris with various size categories including microplastics (1 μm - 5 mm). Alongside the physical presence of microplastics, the release of monomers and additives from plastics into the environment, known as leachates, adds to the complexity of the issue. Both (micro)plastic particles and leachates are of concern. As climate change progresses due to carbon emissions from human activities, ocean warming has emerged as a defining consequence. This second stressor can affect marine organisms at different trophic levels and can alter community compositions.

Within this context, the objective of this study was to assess the effects of plastic leachates on algal growth rates and to explore the combined effects of plastic leachates and ocean warming on copepod mobility. The research began by investigating the ecotoxicity of plastic leachates on *Phaeodactylum tricornutum*, a diatom responsible for oceanic primary production. The results demonstrated growth inhibition caused by PVC leachates and seawater-aged PLA leachates, emphasizing the influence of material weathering on leachates' toxicity. Furthermore, the study delved into the interaction between temperature and plastic leachate toxicity. Surprisingly, despite a 3 °C temperature increase, the toxic effects of PVC leachates on adult *Nitokra spinipes*, a harpacticoid copepod, did not significantly intensify. This outcome underscores the complexity of multiple stressor interactions.

In conclusion, this comprehensive study provides insights to unveil the intricate connections between plastic pollution, ocean warming, and aquatic organisms. By investigating the toxicity of plastic leachates and their interplay with temperature stress, the research contributes to the understanding of potential ecological consequences. Ultimately, this work may guide informed decision-making for sustainable plastic usage and marine ecosystem preservation.

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ACRONYMS

AGM Algal growth medium. 17, 18

ASW Artificial seawater. 15–17

DNSW Diluted natural seawater. 15–17, 19, 55, 58, 60–62

EC_{50} Half maximal effective concentration. ix, 20, 21, 23, 24, 28, 32, 34

Flax-PLA Flax fibers-reinforced polylactic acid. x, xi, 14, 15, 18, 26, 27, 31, 33, 37, 66

IPCC Intergovernmental Panel on Climate Change. 3, 12, 34

ISO International Organization for Standardization. 12, 15–19, 21, 23, 31, 32, 54

MPs Microplastics. 2, 8, 9, 12, 35, 57, 58, 60–62

NPs Nanoplastics. 2, 35

PE Polyethylene. 7, 10

PET Polyethylene terephthalate. 7, 10

PGA Polyglycolic acid. 7

PLA Polylactic acid. 7, 15, 18, 25, 33

PLGA Poly lactic-co-glycolic acid. 7, 57, 59, 60, 62, 63

PP Polypropylene. 7, 10

PS Polystyrene. x, 7, 10, 57–59

PSU Practical salinity unit. 15, 16, 21, 58

PVC Polyvinyl chloride. x, xi, 7, 10, 14–16, 18, 19, 21, 24, 27–29, 31, 32, 34, 37

RCP Representative concentration pathway. ix, 3, 12, 34

rpm Revolutions per minute. 16, 58

SR-PLA Self-reinforced polylactic acid. x, xi, 14, 15, 18, 25–27, 31–33, 37, 66

1. INTRODUCTION

1.1 Plastic pollution

Plastics are one of the most convenient materials to use in a wide range of applications, many of which are used in the packaging industry and for building and construction (PlasticsEurope, 2022). Some of the plastics' main advantages, as opposed to other materials such as paper, wood, glass, or metals, are their high strength-to-weight ratio and durability, as well as their lower unit cost (Andrady and Neal, 2009). This applicability results in a strong and continuing demand for plastics. The global plastic production was equal to 390.7 million tonnes of plastics in 2021 and the production is increasing (Figure 1.1) (PlasticsEurope, 2022).

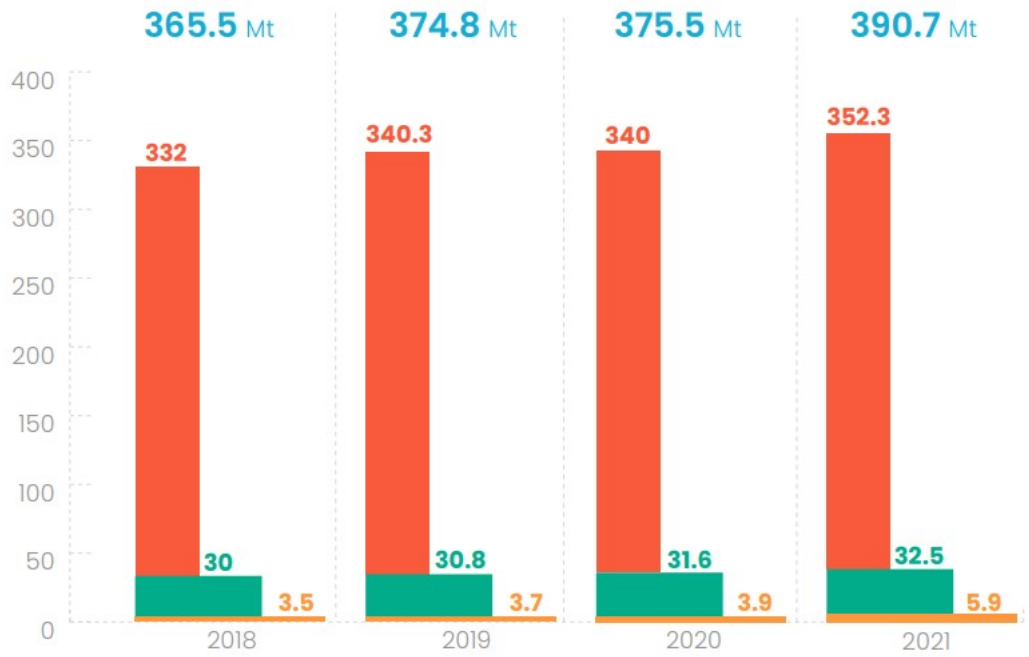


Figure 1.1: World plastic production evolution from 2018 to 2021 in million tonnes. (*Red bars = fossil-based plastics, green bars = post-consumer recycled plastics, and yellow bars = bio-based plastics (including bio-attributed plastics in 2021 data)*) (PlasticsEurope, 2022).

Although there are multiple societal advantages to using plastics, poor waste management and incorrect disposal also lead to issues linked to plastic debris (Andrady and Neal, 2009; Thompson et al., 2009). Plastics enter the natural environment due to direct (accidental) disposal (e.g. littering) or indirectly, through disposal in

dumps or open, uncontrolled landfills. From there, they can be transported by wind, runoff events, waterways, wastewater outflows, currents, and tides, and eventually, end up in marine environments (Jambeck et al., 2015). This includes the occurrence of plastics on beaches, on the seabed, within sediments, buoyant in the water column, and floating on the sea surface (Gallo et al., 2018). This plastic debris can be classified according to size, shape and structure, color, and origin (Hartmann et al., 2019). Different sizes of plastics occur in the environment, where they are exposed to various weathering mechanisms, such as wave action, abrasion, and photo-degradation that continuously fragment the particles. Generally, their size ranges are subdivided into macroplastics (> 20 mm diameter), mesoplastics (5 mm - 20 mm diameter), microplastics (MPs, 1 μm - 5 mm diameter) down to nanoplastics (NPs, < 1 μm diameter) (Franzellitti et al., 2019; Koelmans et al., 2022). The MPs formed in this way are referred to as secondary MPs (Boucher and Friot, 2017). Primary MPs on the other hand, are MPs that are manufactured and directly released into the environment in the form of beads and pellets. They originate from multiple industrial and domestic applications such as paints, scrubbers, and cosmetic and personal healthcare products (Boucher and Friot, 2017; SAPEA, 2019; UNEP, 2016). The smaller their size, the higher the potential for uptake by organisms, which makes it an environmental issue of concern (Catarino et al., 2021).

Another source of concern associated with plastic debris is the leaching of monomers and additives from the polymer matrix into the environment (Gunaalan et al., 2020). These mixtures are referred to as 'leachates' and may contain ecotoxic components (Delaeter et al., 2022; Koelmans et al., 2014). Additives are chemicals that are associated with the polymers to modify the properties of the finished item (e.g. plasticizers to increase flexibility) and can thereby enhance the performance of the plastic (Andrady and Neal, 2009). Some of these leached chemicals such as bisphenol A and phthalates have long been known to induce toxicity in marine organisms (Kang et al., 2007; Zhang et al., 2021a). MPs as well as leachates from plastic pollution raise important concerns about their potential for toxicological effects on marine organisms, which are generally assessed using high concentrations as a proof-of-concept approach (Delaeter et al., 2022; Huvet et al., 2016). However, organisms are exposed to a multitude of stressors in their environment beyond plastic debris, and their joint effects are mostly unknown (Catarino et al., 2022).

1.2 Ocean warming

Climate change is an important human-induced environmental stressor that has multifaceted consequences, affecting various aspects of the environment, societies, and economies worldwide. Regarding marine environments, anthropogenic carbon emissions are causing worldwide trends of ocean warming, increasing occurrence, intensity, and duration of ocean heatwaves, ocean acidification, sea-level

rise, and ocean deoxygenation (Cooley et al., 2022). All these trends affect marine systems and have cascading effects (e.g. impacts on nutrient cycles and primary production) resulting in multiple societal consequences such as impacts on fisheries with implications for food production (Magnan and Gattuso, 2016). This report focuses on ocean warming as a consequence of climate change, which is an essential part of the planet's energy distribution. Carbon emissions from human activities, mainly the burning of fossil fuels, increase the concentration of greenhouse gases in the atmosphere. These gases enhance the natural greenhouse effect, trapping more heat and warming the planet, including the oceans (Shakun et al., 2012). Bindoff et al. (2019) indicated that there is high confidence that ocean warming dominated the rise in the Earth's energy inventory from 1971 to 2010. Fox-Kemper et al. (2021) confirmed that 91% of the total energy gained from 1971 to 2018 was stored in the ocean. By 2100, the global sea surface temperature is predicted to increase 3 °C according to RCP8.5 (i.e., "business as usual") of the IPCC (Figure 1.2) (Abram et al., 2019; Bindoff et al., 2019). Rising temperatures affect marine organisms at multiple trophic levels, and can thereby change community compositions (Bindoff et al., 2019). Ocean warming, an omnipresent environmental stressor, adds an intriguing dimension to research on marine organisms that aims to evaluate the future impacts of various stressors. To unravel the intricate dynamics of plastic debris and ocean warming, researchers can employ model species that offer insights into the complex and multifaceted responses to these stressors.

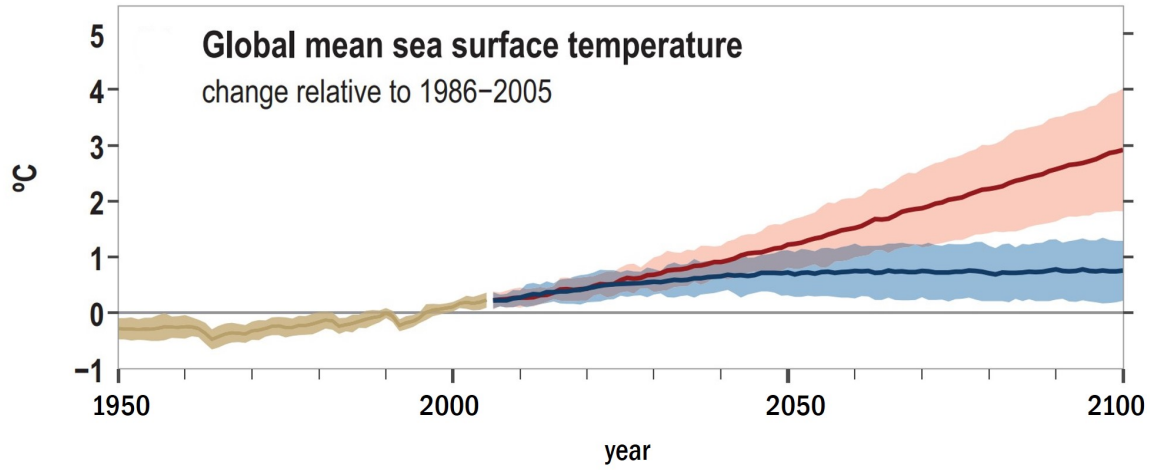
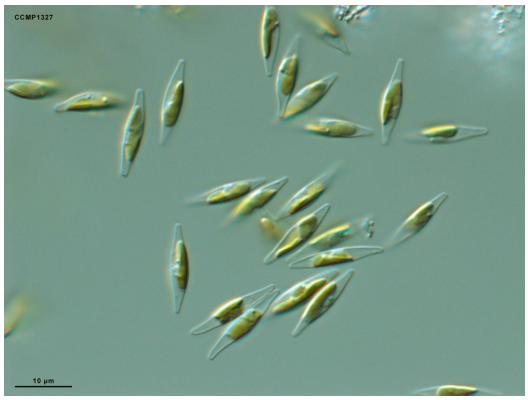


Figure 1.2: Past and future changes in the global mean sea surface temperature *(brown = historically (modelled), blue = projected RCP2.6, red = projected RCP8.5)* (Abram et al., 2019)

1.3 Characteristics of the model species

Two model species, *Phaeodactylum tricornutum* and *Nitokra spinipes* were considered in this work to perform ecotoxicological tests (Figure 1.3).



(a)

(b)

Figure 1.3: Pictures of model species (a) *Phaeodactylum tricornutum* under a light microscope (DM1000, Leica) with 10x objective lens *(scale bar = 10 µm)* and (b) *Nitokra spinipes* under a light microscope (DM1000, Leica) with 10x objective lens *(scale bar = 200 µm)*.

As a diatom, *P. tricornutum* belongs to a group of organisms that is responsible for about 40% of the oceanic primary production (Nelson et al., 1995). They generate vast amounts of organic material that sustain marine ecosystems and contribute to the Earth's carbon cycle, while also playing important roles in the biogeochemical cycling of nutrients like nitrogen and silicon (Armbrust, 2009; Bowler et al., 2010). They are thus of importance for the functioning of our planet's ecosystems (Falkowski, 2015). *Phaeodactylum tricornutum* is a phytoplanktonic primary producer that is known for its ease of cultivation and short generation time, making it a widely used model species (De Martino et al., 2007; Scala et al., 2002).

The second model species, *N. spinipes* is a harpacticoid copepod. It is a primary consumer that connects primary producers to higher trophic levels in the food web (Kwok et al., 2015). Copepods are a group of small crustaceans that can be found in almost all aquatic environments. In the marine environment, copepods play an important role in the carbon budget and nutrient cycles through daily vertical migrations and carbon and nitrogen transfers into the deep sea in the form of sinking carcasses and fecal pellets (Frangoulis et al., 2004, 2011). *Nitokra spinipes* have life cycle stages between which they undergo metamorphoses, which is helpful to distinguish them: *nauplii*, copepodites, and adults. We can thus trace their development, which is interesting to perform toxicity tests on specific life-cycle stages and full life-cycle toxicity tests, possibly demonstrating differential fitness and/or cumulative impacts in response to toxic stressors (Hammers-Wirtz and Ratte, 2000). The species has a long history of toxicity assessments, starting with research conducted by Bengtsson (1978). These typical characteristics together with the fact that both species are abundant at the base of the food chain and may accidentally uptake MPs and/or plastic leachates make *P. tricornutum* and *N. spinipes* interesting model species.

In conclusion, this research sheds light on two human-induced environmental stressors: plastic waste and ocean warming. The investigation of (combined) effects of these stressors calls for the use of model species such as *P. tricornutum* and *N. spinipes*, which provide insights into ecotoxicological responses. As we investigate the consequences of plastic debris and rising ocean temperatures, these model species will be critical in understanding the complex dynamics that will underpin marine ecosystem health in the future.

2. LITERATURE REVIEW

2.1 Occurrence of microplastics and plastic leachates in aquatic environments

Plastics are made by mixing a polymer with a complex blend of additives that improve the desirable properties of the finished product. They come in different sizes, shapes, and colors and are classified based on their chemical structure, polarity, or application (Gunaalan et al., 2020). Numerous polymers and additives exist, each stemming from the unique properties and requirements of various applications, industries, and technologies. The most abundant polymers, dominating the market are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), and polyethylene terephthalate (PET) (Hidalgo-Ruz et al., 2012; PlasticsEurope, 2022). For the production of these plastics, fossil-based feedstocks are used that are derived from natural gas processing or crude oil refining. Fossil-based plastics consist the largest part of plastic production (90.2%) (Gunaalan et al., 2020; PlasticsEurope, 2022). The remaining part of plastic production consists of post-consumer recycled plastics (8.3%) and bio-based/bioattributed plastics (1.5%) (PlasticsEurope, 2022). Developing synthetic biodegradable polymers such as polylactic acid (PLA), polyglycolic acid (PGA), and poly lactic-co-glycolic acid (PLGA) that can be decomposed by microorganisms is important to make plastic waste management strategies more effective and ecologically sound (Gurunathan et al., 2015; Lim and Thian, 2022). To make sure biobased composites are as eco-friendly as they are believed to be, they still need to be studied further in ecotoxicology, since currently, the real risks of both their derived MPs and complex leachates mixtures on biota are largely unknown (Curto et al., 2021; Iwata, 2015). Since plastic pollution raises concerns about the environment and sustainability issues, there is an incentive to improve the biodegradability of plastics and make them non-toxic (Bhagabati, 2020; La Mantia and Morreale, 2011; Netravali and Chabba, 2003). However, this does not allow us to dismiss problems from persistent or non-biodegradable plastic pollution.

Because the carrying capacities of the different transportation methods of plastics depend on local weather conditions and watershed characteristics as well as the properties of the plastics, it is difficult to extrapolate plastic waste inputs from land into oceans on a global scale (Jambeck et al., 2015; Lebreton et al., 2017). To give an idea of the order of magnitude of the pollution problem, Jambeck et al. (2015)

estimated 4.8 to 12.7 million tonnes of plastic debris entered the ocean in 2010. Later, Borrelle et al. (2020) estimated that in 2016 the amount of plastic waste entering the aquatic ecosystems was about 19 to 23 million tonnes. Concentration measurements show that MPs are more numerically abundant than macro- or mesoplastics and that they generally end up in sediments as a final sink (Gunaalan et al., 2020).

In recent years, many studies aimed to find out how and in what concentrations MPs and leachates are distributed in the marine environment (Gunaalan et al., 2020; Ioakeimidis et al., 2014). Estimating the environmental concentrations of MPs is challenging and requires innovative highly expert techniques because of their spatiotemporal variability and because of their properties making them difficult to distinguish from other particles (Filella, 2015; Hidalgo-Ruz et al., 2012). Bucci et al. (2020) estimated a median of $1.4 \cdot 10^{-1}$ particles / L out of 137 reported MPs concentrations ranging from $2.80 \cdot 10^{-5}$ particles / L to $1.77 \cdot 10^{3}$ particles / L. Lenz et al. (2016) related field concentrations to particle sizes, resulting in a regression line: $x = 3{,}188 \cdot y^{-2.67}$, with x the concentration (particles / L) and y the particle size (μm). They found that MPs occur in the environment at concentrations of about 1 ng / L to 1 μg / L.

As for leachates, De Frond et al. (2019) estimated that approximately 190 tonnes of twenty chemical additives entered the ocean along with 87,000 tonnes of plastic beach litter in 2015, which they expect to be an underestimation of the global amount of leachates entering the marine environment. Jang et al. (2017) focused on recording additives from expanded polystyrene fragments and found maximal concentrations of hexabromocyclododecane (HBCDs) reaching levels of 3350 ± 5860 ng / g. We know that the amount of leachates is relative to the amount of plastics entering the oceans and that weathering forces can stimulate leaching along with plastic fragmentation (Bejgarn et al., 2015; Gunaalan et al., 2020). Overall, the complexity of microplastic and leachate mixtures and the uncertainties about their concentrations make scientific risk assessments a hard task (Catarino et al., 2021). To provide environmentally relevant data regarding the effects of MPs and plastic leachates on marine organisms, we need more observations on realistic concentrations, shapes, and sizes of plastics in the environment.

2.2 Studied effects of microplastics on marine organisms

Despite the uncertainties on environmental MPs concentrations, it is generally believed that marine organisms are increasingly exposed to these particles. Studies show that organisms can ingest microplastic particles and that the smaller the particles get, the wider the range of organisms that can ingest them gets (Beiras et al.,

2018; Besseling et al., 2013; Cole and Galloway, 2015; Cózar et al., 2014; Desforges et al., 2015; Lee et al., 2013; Sun et al., 2017; Sussarellu et al., 2016; von Moos et al., 2012; Wang et al., 2019; Zhang et al., 2021b). In some cases, high ingestion rates have led to the accumulation of microplastics in the digestive tracts of these organisms (Gambardella et al., 2017; Jeyavani et al., 2022). Microplastic ingestion has been linked to various physiological impacts on aquatic organisms. The particles may cause blockages in the digestive system, reducing nutrient absorption, and leading to malnutrition and decreases in energy reserves (Besseling et al., 2013; Wright et al., 2013). Zhang et al. (2017) showed photosynthesis in marine microalgae can be reduced when exposed to MPs. Additionally, microplastics have been shown to cause oxidative stress and inflammation in certain species (Gambardella et al., 2017; Jeyavani et al., 2022). Oxidative stress can lead to cellular damage and impact the overall health and functioning of an organism (Jeyavani et al., 2022). Some studies suggest that MPs can cause hormonal disruption, which can affect growth, reproduction, and development (Jeyavani et al., 2022; Sjollema et al., 2016; Sussarellu et al., 2016; Zhang et al., 2017). Studies have also reported changes in activity patterns such as altered feeding behavior (Green et al., 2016; Wang et al., 2019), and reduced mobility (Gambardella et al., 2017; Jeyavani et al., 2022). Reduced consumption of natural food sources can lead to decreased energy intake, affecting an organism's fitness, and can impact nutrient cycling within ecosystems, altering the availability of nutrients for other organisms in the food web. When mobility is reduced, a predator's ability to capture prey could be compromised, affecting the entire predator-prey balance in the ecosystem. These behavioral impacts thus potentially have cascading effects on population dynamics (Wright et al., 2013). Additionally, MPs can move through marine food webs via trophic transfer (Cedervall et al., 2012; Murray and Cowie, 2011; Setälä et al., 2014). The presence of MPs in marine food sources has been shown, which may ultimately become of concern for human health (Bouwmeester et al., 2015; Diepens and Koelmans, 2018; Galloway, 2015; Van Cauwenberghe et al., 2015). However, the consequences of microplastic exposure are not straightforward. Most of these findings were based on relatively high microplastic concentrations and it is important to mention that some studies also found that there were no acute toxic effects, no physiological effects, and/or no behavioral effects observed when exposing organisms to microplastics (Beiras et al., 2018; Cole and Galloway, 2015; Lee et al., 2013; Lo and Chan, 2018; Niu et al., 2021; Sjollema et al., 2016). Although exposure of aquatic organisms to MPs is confirmed, indications of hazardous effects are inconsistent and need further research.

2.3 Studied effects of plastic leachates on marine organisms

It is widely assumed that marine organisms are increasingly exposed to leachates from plastic pollution. The observed effects of plastic leachates depend on many factors that influence the potential exposure and subsequently their toxicity such as the test species, polymer type, chemical composition, accumulated contaminants, and weathering status of the plastic (Delaeter et al., 2022; Koelmans et al., 2014). Numerous studies have been conducted to understand the effects of plastic leachates on various aspects of marine life. Induction of oxidative stress by plastic leachates was assessed by investigating biomarkers in the mussel *Mytilus galloprovincialis* (Capolupo et al., 2021). These biomarkers serve as the first signals of biological effects induced by toxicants. Oxidative stress was reported in these organisms and the severity of the stress was dependent on the type of polymer used to make the leachates (Capolupo et al., 2021). Of the studied plastics, leachates from PP, PVC, and car tire rubber (CTR) were assigned to moderate stress effects, followed by PS, having a low stress effect and eventually PET, resulting in a healthy status of *M. galloprovincialis* (Capolupo et al., 2021). Moreover, plastic leachates' impact on embryonic and larval development was studied, particularly in echinoderms and mollusks. Leachates from certain plastics, like PE, PET, PVC, and PP caused abnormal embryonic and/or larval development in sea urchins (Cormier et al., 2021; Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Piccardo et al., 2020; Rendell-Bhatti et al., 2021) and clams, oysters, and mussels (Capolupo et al., 2020; Gardon et al., 2020; Ke et al., 2019). Additionally, the reproductive impacts of plastic leachates were investigated by considering parameters like egg production, hatching, and fertilization. The effects on reproduction were found to be dependent on the type of polymer, species, and leachates concentrations used in the studies (Capolupo et al., 2020; Ke et al., 2019; Koski et al., 2021; Li et al., 2016; Thomas et al., 2020). Mortality is another extensively studied parameter concerning plastic leachates' impact on marine organisms, both on larvae and adults. Exposure to plastic leachates increased mortality in barnacle and crustacean larvae (Li et al., 2016; Trestrail et al., 2020). In adult mussels, mortality increased when exposed to certain plastic leachates, such as PP, PVC, and CTR, while PET and PS leachates did not exhibit similar effects (Capolupo et al., 2020). The survival of adult copepods to plastic leachates varied depending on the polymer type, additives composition, and weathering processes (Bejgarn et al., 2015; Gewert et al., 2021; Lehtiniemi et al., 2021). It was observed that weathering, such as artificial UV exposure, could trigger toxicity in plastics that were not toxic before the process or could make them lose their toxicity (Bejgarn et al., 2015; Gewert et al., 2021). Furthermore, plastic leachates have been found to influence behavior and cognition in marine organisms. For instance, mussel species exhibited species-specific changes in aggregation behavior and anti-predator behaviors when

exposed to specific plastic leachates (Seuront et al., 2021). Overall, it was observed that toxicity can be influenced by the cocktail of additives and pollutants released during the leaching process, as well as the polymer's origin and the species that is exposed to the substance (Nobre et al., 2015). Understanding the diverse impacts of different plastics, additives, concentrations, and weathering processes is crucial for mitigating the harmful effects of plastic pollution on marine life. More research is needed to comprehensively address this environmental challenge and protect marine ecosystems.

2.4 Multiple stressors approach

To assess realistic effects of MPs and leachates on biota, it is important to include additional stressors arising from climate change such as ocean warming, marine heat waves, ocean acidification, and deoxygenation in assessments of plastic pollution effects (Catarino et al., 2022; Cooley et al., 2022). In this context we characterize a stressor as any biotic or abiotic factor that brings about noticeable impacts (whether positive or negative) on individual organisms, the composition of communities, or the operational dynamics of ecosystems (i.e. biological systems) by surpassing its typical range of fluctuations (Piggott et al., 2015; Segner et al., 2014; Van Straalen, 2003). The impact of a stressor is contingent upon factors such as its intensity, timing, and duration, in addition to the physiological or ecological characteristics of the recipient (Segner et al., 2014). Biological systems in our ocean are more and more exposed to multiple stressors simultaneously. When multiple stressors come into play, the biological system may respond differently than when it was only exposed to one of these stressors. This is because the stressors could combine additively, synergistically, or antagonistically (Piggott et al., 2015; Griffen et al., 2016). For this reason, it is important to include multiple stressors in experimental work with biological systems after collecting background information on their single-stressor responses (Griffen et al., 2016). Looking at ocean warming as a single stressor, it is expected to affect metabolic rates, respiration, photosynthesis, growth, pigment contents, and antioxidant systems (Cooley et al., 2022; Gomes and Juneau, 2017; Regaudie-de Gioux and Duarte, 2012).

When combining ocean warming and plastic pollution exposure, we get new insights into the combined responses of biological systems. Kratina et al. (2019) found a decrease in the metabolic rate of *Gammarus pulex* when simultaneously exposing the species to higher temperatures and MPs. Exposing *Daphnia magna* to elevated temperature treatments led to accelerated ingestion of MPs, resulting in abnormalities in intestinal epithelial cells and changes in energy allocation (Lyu et al., 2021). Experimental exposure of the cyanobacterium *Synechococcus* sp. to MPs additional to ocean warming weakened the warming-driven enhancement of cell growth and carbon fixation and intensified the reduction of photosynthesis pig-

ment contents (Zeng et al., 2023). On the other hand, Weber et al. (2020) did not detect interaction between microplastic exposure and temperature stress in *Dreissena polymorpha* mussels, despite observing separate effects on energy reserves, oxidative stress, and immune function when the stressors were tested individually. Reichert et al. (2021) observed that certain species of reef-building corals displayed an intensified response to thermal stress upon exposure to MPs, while others exhibited a mitigating effect. These variations underscore the substantial diversity in sensitivity to multiple stressors. The current and future consequences of the co-occurrence of plastic pollution and warming in marine ecosystems are still largely unexplored (Ford et al., 2022). Broadening the scope of plastic pollution effect evaluations under current and future environmental circumstances necessitates active participation of the research community in order to provide stakeholders with information on the vulnerability of marine species and ecosystems (Catarino et al., 2022).

2.5 Research objectives

This study had two main goals: 1) to assess the effects of plastic leachates, resulting from plastic pollution, on *P. tricornutum*; and 2) to assess the combined effects of ocean warming and plastic leachates on *N. spinipes*.

For the first goal, *P. tricornutum* were exposed to plastic leachates and the ecotoxicity of these leachates was assessed by studying if this exposure affected the specific growth rate of the algae after 72 hours. Both petroleum- and bio-based plastics were taken into account for this assessment in order to acquire further insights into the sustainability of future biocomposite applications and possibly support a transition process to improved plastic materials.

For the second objective, *N. spinipes* adults were exposed to different types and concentrations of plastic leachate solutions at two temperatures. A temperature difference of 3 °C was chosen, which reflects the predicted global sea surface warming according to RCP8.5 of the IPCC (Abram et al., 2019; Bindoff et al., 2019). It was assessed if the combined effects of this temperature rise and plastic leachates affected the survival of the copepod species. This is key to understanding potential repercussions at a population level. All assessments were done in laboratory experiments following standardized ISO protocols (Table 2.1).

Additionally, further method development was done to assess if the harpacticoid copepod species ingested spherical beads of MPs, and trial tests were performed to gain insights into possible sub-lethal effects of MPs on *N. spinipes*. This method development can be found in Appendix B and will be included in future studies.

Our first hypothesis was that different plastic leachates would have different ecotoxicities. For the second goal, the hypothesis was that temperature stress could further impact the toxicological effects of plastic leachates on *N. spinipes* species.

Table 2.1: Overview of performed tests to assess the ecotoxicity of plastic leachates on model species. All leachates solution concentrations are dilutions from a leachates stock solution with a solid-to-liquid ratio of 80 g / L. Tests performed with two temperatures aimed to assess the combined effects of temperature rise and plastic leachates.

Test organism	**Leachates solution**			**Tempera-ture**	**Experimental duration and exposure method**
	Polymer type	**Leaching time**	**Concentration**		
Phaeodactylum tricornutum	pristine PVC	21 d	90 % v / v	18 ± 2 °C	72 hours in artificial seawater with algal nutrient stocks (ISO, 2016a)
Phaeodactylum tricornutum	pristine SR-PLA and Flax-PLA	21 d	10, 24 and 60 % v / v	18 ± 2 °C	72 hours in artificial seawater with algal nutrient stocks (ISO, 2016a)
Phaeodactylum tricornutum	seawater-aged SR-PLA and Flax-PLA	21 d	10, 24 and 60 % v / v	18 ± 2 °C	72 hours in artificial seawater with algal nutrient stocks (ISO, 2016a)
Nitokra spinipes	pristine PVC	7 d	21, 42.2, 56.25, 75 and 100 % v / v	22 ± 1 °C and 25 ± 1 °C	96 hours in diluted natu-ral seawater (ISO, 1999)
Nitokra spinipes	seawater-aged PVC	7 d	21, 42.2, 56.25, 75 and 100 % v / v	22 ± 1 °C and 25 ± 1 °C	96 hours in diluted natu-ral seawater (ISO, 1999)

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Seawater

Artificial seawater (ASW) of 33 PSU was made by adding NaCl, $MgCl_2 \cdot 6H_2O$, Na_2SO_4 (anhydrous), $CaCl_2$ (anhydrous), KCl, $NaHCO_3$ and H_3BO_3 to Milli-Q water (Millipore Corporation) as mentioned in protocol 10253 of the International Organization for Standardization (ISO) (ISO, 2016a). ASW was filtered trough a 0.2 μm membrane filter (Sterivex™) and kept in dark at 4 °C in autoclave-sterilized bottles (Steam sterilizer Laboklav 25: SHP Steriltechnik AG; glass bottles: DURAN®).

Diluted natural seawater (DNSW) was prepared by mixing natural seawater that was pumped from the North Sea (Ostend, Belgium) with Milli-Q water (Millipore Corporation) to obtain a salinity of 7 PSU for culturing and testing the model species *Nitokra spinipes*.

Salinity was checked with a refractometer (HANNA® Intruments). The pH of every ASW and DNSW batch was checked with a pH meter (PCE Instruments) and adjusted to 8.0 ± 0.2 (National Institute of Standards and Technology (NIST) scale) with HCl or NaOH.

3.1.2 PVC and PLA leachates stock solutions for algal growth inhibition tests

To prepare leachates stock solutions, we first cut plastic flakes of the selected polymer types pristine PVC and PLA. Pristine PVC was sawed from a grey sanitary tube (Scala Plastics) into 2 x 2 x 0.18 cm^3 flakes.Two kinds of PLA plastics, pristine PLA-co-PLA (SR-PLA) and Flax-co-PLA (Flax-PLA), were cut into 2 x 4 x 0.3 cm^3 flakes using a high-pressure water knife. To mimick environmental weathering, part of the SR-PLA and Flax-PLA flakes were immersed in temperature-controlled natural seawater tanks at IFREMER (France). The seawater in the tanks was pumped from the Brest Estuary (France) and was continuously renewed and kept at 25 ± 2 °C. These preparations resulted in one type of pristine PVC flakes and four different types of PLA flakes: pristine SR-PLA, pristine Flax-PLA, seawater-aged SR-PLA and seawater-

aged Flax-PLA. All flakes were incubated (Heratherm™) at 22 ± 1 °C in dark until dry before the preparation of the leachates.

All leachates stock solutions were made in ASW. Following Capolupo et al. (2020), solid-to-liquid ratios of 80 g / L plastic flakes were added to 320 mL ASW in pre-sterilized 500 mL Erlenmeyer flasks, wrapped in aluminium foil. The samples were closed off with cotton wool and incubated on an orbital shaker (Edmund Bühler GmbH) with a speed of 80 rpm at room temperature (22 ± 1 °C) for 21 days. Afterward, the leachates stock solutions were filtered through a 0.2 μm regenerated cellulose membrane filter (Whatman™), to an end volume of 320 mL per treatment. They were stored in dark at -22 °C in a laboratory freezer (Liebherr).

The filtered leachates stock solutions were supplemented with algal nutrients stocks and/or diluted with more ASW to perform the different algal growth inhibition tests (Section 3.3) according to ISO 10253 protocol (ISO, 2016a). This resulted in plastic leachate exposure concentrations smaller than 80 g / L, as represented in Table 2.1. Because of the complexity of leachates, the concentrations are given in volumetric percentages (% v / v) instead of absolute values.

3.1.3 PVC leachates stock solutions for copepod acute lethal toxicity tests

Leachates stock solutions for copepod acute lethal toxicity tests were made with 1 x 1 x 1 cm^3 pristine and seawater-aged PVC flakes. The pristine PVC flakes were directly sawed from rinsed grey PVC litter items, while the seawater-aged PVC flakes were made by suspending 15 x 15 cm sanded grey PVC in seawater (harbour of Ostend, Belgium) for two months before sawing them into pieces. Solid-to-liquid ratios of 80 g / L plastic flakes were added to 100 mL DNSW of 7 PSU in pre-sterilized 250 mL bottles (DURAN®). The bottles were capped with a Teflon cap (Griffon®) and Parafilm M (Bemis™) and were put on an orbital shaker (Edmund Bühler GmbH) in dark (aluminium foil wrap) with a speed of 80 rpm at room temperature (22 ± 1 °C) for seven days. Afterwards, the solutions were filtered through a 0.2 μm cellulose filter (Whatman™) and stored in dark at -22 °C. Again, concentrations of dilutions of the complex leachates stock solutions used to perform the ecotoxicity tests are given in nominal values (% v / v) (Table 2.1).

3.2 Maintenance of model species

Two model species were used: a marine diatom, *Phaeodactylum tricornutum* and a harpacticoid copepod, *Nitokra spinipes*.

The *P. tricornutum* strain 1052/1A was obtained from the Culture Collection of Algae and Protozoa (Oban, United Kingdom) and 100 mL cultures were made in presterilized 250 mL Erlenmeyer flasks at 18 ± 2 °C under a continuous white light (> 2,500 lux), and manually shaken twice a day. Algal growth medium (AGM) was prepared by adding nutrients stock solutions to ASW (Subsection 3.1.1) and was filtered through a 0.2 μm sterile filter (Sterivex™) as describes by ISO (2016a).

The strain of *N. spinipes* was isolated from sediments in the Tvaren Bay, Baltic Sea in 1975 and has been in continuous culture ever since. According to ISO protocol 14669, the culture medium was prepared in DNSW (Subsection 3.1.1) and filtered through a 0.2 μm sterile filter (Sterivex™) (ISO, 1999). *Nitokra spinipes* were acclimatized to two different temperatures (22 °C and 25 °C) in darkness in two incubators (Heratherm™). They were kept in 150 mL crystallization dishes (DURAN®) under static-renewal conditions and fed weekly with a commercial dried salmon feed (Astra-EWOS®). To minimize evaporation losses while incubated, the dishes were covered with pierced plastic Petri dishes.

3.3 Algal growth inhibition tests

3.3.1 General algal growth inhibition test method

All 72-h algal growth inhibition tests were performed on *Phaeodactylum tricornutum* species and followed the ISO 10253 protocol (ISO, 2016a). Four days prior to each test, a pre-culture was made and incubated at conditions mentioned in Section 3.2 to obtain exponentially growing *P. tricornutum* (exponential growth explained in Appendix A.2). During the 72-h test, all flasks were again incubated at these conditions, but additionally, they were gently bubbled with air through a syringe filter (Satorius Minisart®). Every 24 ± 2 h, including timepoint 0 h, the cell density (cells / mL) was quantified in each flask. *Phaeodactylum tricornutum* cell densities were determined with a hemocytometer under a light microscope (DM1000, Leica Microsystems) with 10x objective lens (details explained in Appendix A.1). The interpretation of these cell density measurements is discussed in Subsection 3.5.1. The positions of the flasks were randomly switched after each cell density counting.

3.3.2 Quality control test

To check the repeatability of the growth inhibition test, first, a toxicity test was performed with the chemical potassium dichromate ($K_2Cr_2O_7$), as mentioned in Annex B of the ISO 10253 protocol (ISO, 2016a). A dilution series was made in AGM (Section 3.2) resulting in five $K_2Cr_2O_7$ concentrations 40 mg / L, 20 mg / L, 10 mg / L, 5 mg / L, and 2.5 mg / L, named treatments C5, C4, C3, C2, and C1 respectively.

Three replicates with 100 mL of these concentrations and six 100 mL controls (treatment C0, AGM without $K_2Cr_2O_7$) were added to pre-sterilized 250 mL Erlenmeyer flasks. *P. tricornutum* cells of a pre-culture were then added to result in an initial cell density of about $1 \cdot 10^4$ cells / mL in each flask. The pH of the controls was measured at the beginning and at the end of the quality control test as part of a validity criterium (Appendix C.1.1).

3.3.3 Ecotoxicology of pristine PVC leachates

The ecotoxicity of 90 % v / v pristine PVC leachates was evaluated. In this case, five replicates of AGM with 90 % v / v pristine PVC leachates (Subsection 3.1.2) and five control replicates (AGM without plastic leachates) of 100 mL with an initial *P. tricornutum* cell density of about $1 \cdot 10^4$ cells / mL were made in pre-sterilized 250 mL Erlenmeyer flasks.

3.3.4 Ecotoxicology of PLA leachates

The 72-h algal growth inhibition test with four types of PLA leachates was performed in two independent batches. One batch was made for the pristine SR-PLA and Flax-PLA and another one for the seawater-aged SR-PLA and Flax-PLA leachates stock solutions (Subsection 3.1.2). Each PLA leachates stock solution was diluted in AGM to create three treatments with volumetric concentrations of 10 % v / v, 24 % v / v and 60 % v / v. Three replicates of each treatment and a total of six replicates of control treatments (AGM only) per batch were made with an initial algal cell density of approximately $3 \cdot 10^4$ cells / mL.

3.4 Copepod acute lethal toxicity tests

3.4.1 General acute lethal toxicity test method

Acute lethal toxicity tests were performed on salmon-fed *N. spinipes* adults of three to four weeks old following the ISO 14669 protocol (ISO, 1999). During these tests, the copepods were exposed to a range of leachates concentrations in 10 mL pre-rinsed glass vials. In ISO protocol 14669, a copepod is considered dead when it does not swim nor show appendage movements within an observation period of 10 s. To avoid confusion about the findings of this research, we refer to immobility rather than mortality for these conditions. To check if the test method was valid the immobility of the test controls had to be less than or equal to 10 %, and the toxicity of a reference chemical was confirmed to be within the expected range (Subsection

3.4.2). For all tests, immobility was recorded under a stereo microscope (S8APO, Leica Microsystems) after 96 h.

3.4.2 Quality control test

Before testing the ecotoxicity of plastic leachates, a quality control test with *N. spinipes* was performed at 22 ± 1 °C with a dilution series of potassium dichromate ($K_2Cr_2O_7$). This is part of the ISO 14669 protocol (ISO, 1999). The control temperature of 22 ± 1 °C was chosen since the reference tests in the protocol were performed at 20 ± 2 °C. A $K_2Cr_2O_7$ solution of 80 mg / L was made in DNSW (Subsection 3.1.1). This solution was diluted with DNSW to result in six treatments containing the reference substance: 80, 40, 20, 10, 5, and 2.5 mg / L $K_2Cr_2O_7$. Additionally, one control treatment without $K_2Cr_2O_7$ (only DNSW) was made. Four replicates were prepared for each of the treatments. Specifically, for every nominal concentration (treatment), four 10 mL glass vials were filled with five *N. spinipes* adults in 2.5 mL of the appropriate solution. To make sure unwanted larvae, juveniles, and fecal pellets were excluded from the test, the adults were separated from the culture before adding them to the vials. The vials were then put in dark in an incubator (Heratherm™) at the appropriate temperature (22 ± 1 °C) for 96 h. After 96 h, the number of immobile copepods in each vial was recorded as described in Subsection 3.4.1.

3.4.3 Ecotoxicology of pristine and environmental PVC leachates at 22 °C and 25 °C

Pristine and seawater-aged PVC were both chosen to perform acute lethal toxicity tests at 22 ±1 °C and 25 ±1 °C. Before each test, the leachates stock solutions (Subsection 3.1.3) and DNSW (Subsection 3.1.1) were incubated (Heratherm™) in dark at the test temperature for 24 h. To assure comparability between the four separate tests, they were all performed with the same leachate concentrations. Dilution series of 21.09, 42.19, 56.25, 75.00 and 100.0 % v / v were made in DNSW from each leachates stock solution. Again, four replicates of vials with five copepods added to 2.5 mL of the different leachates solutions and four controls were made per test. The vials were incubated (Heratherm™) in dark at the appropriate temperature for 96 h and the number of immobile copepods was recorded as described in Subsection 3.4.1.

3.5 Data analysis

3.5.1 Algal cell density data analysis

Based on the *P. tricornutum* cell density (cells / mL) measurements in the algal growth inhibition tests, the following data analysis was done in R v4.2.3 (R Core Team, 2021). To check if exponential growth in the control flasks took place during the test, growth curves were generated as the logarithm of the mean cell density against time (d). The average specific growth rates μ (d^{-1}) after 72 h (= 3 d) were then calculated for each test vessel using Equation 3.1:

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0} \tag{3.1}$$

where N_0 (cells / mL) and N_L (cells / mL) are the cell densities at respectively t_0 (d) time of the start and t_L (d) time of test termination. For each test, $\overline{\mu}_c$ (d^{-1}) the mean value of the average specific growth rates of the control flasks after 72 h was calculated. To assess the influence of the plastic leachates on the algal growth rate, multiple statistical tests were done with the observed average specific growth rates after 72 h. Statistical significance was accepted when $p < 0.05$ for all tests. First, a one-way ANOVA was performed to check for any significant differences between the average specific growth rates of all different treatments. To perform this test, normality, and homoscedasticity conditions were checked with a Shapiro-Wilk test (stats package) and a Levene test (car package) respectively (Appendices C.1.2 and C.1.3). Afterward, a post-hoc Dunnett test was performed to assess the differences between the mean of each treatment's average specific growth rates and the $\overline{\mu}_c$ control value.

For the quality control test, the EC_{50} value was calculated, i.e., the concentration of $K_2Cr_2O_7$ (mg / L) that results in a 50 % reduction in specific growth rate, relative to the controls. The average specific growth rates after 72 h of exposure to the highest concentration of $K_2Cr_2O_7$ (40 mg / L) were excluded from the following calculations because they were negative values. The $\overline{\mu}_c$ value was used to calculate the percentage of algal growth inhibition, $I_{\mu i}$ (%) for each individual test flask i using Equation 3.2:

$$I_{\mu i} = \frac{\overline{\mu}_c - \mu_i}{\overline{\mu}_c} \cdot 100 \tag{3.2}$$

where μ_i is the average specific growth rate after 72 h for test flask i. For each group of test flasks in which the algae were exposed to the same concentration of $K_2Cr_2O_7$, the mean and standard deviation of the algal growth inhibitions (%) were calculated. Based on these values a four-parameter log-logistic curve was fitted using the 'drm' function of the R software 'drc' package to describe the relationship between the percentage of algal growth inhibition (%) and the concentration of the

chemical substance in the algal growth medium (Ritz et al., 2015). The effective concentration EC_{50} was then determined using the 'ED' function of the R software 'drc' package (Ritz et al., 2015). Based on this 72 h EC_{50} value of $K_2Cr_2O_7$, the repeatability of the test was evaluated using Annex B of the ISO 10253 protocol (ISO, 2016a).

3.5.2 Copepod immobility data analysis

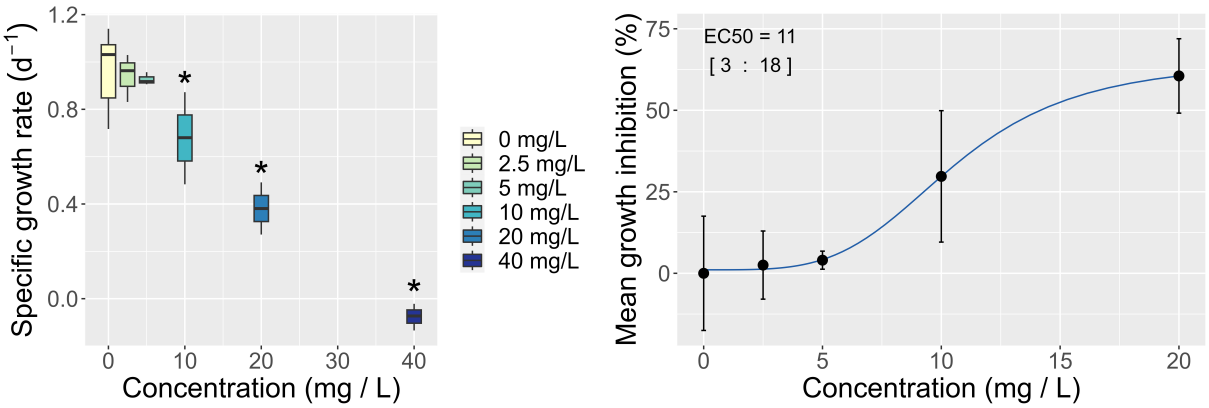
The collected data on the number of immobile copepods after 96 h of exposure to dilution series of test substances ($K_2Cr_2O_7$ and pristine and seawater-aged PVC leachate solutions) at 22 ± 1 °C and 25 ± 1 °C was processed in R v4.2.3 (R Core Team, 2021). The number of immobile copepods that were exposed to the same treatment (replicates) were pooled and percentages of immobility after 96 h were calculated. The immobility (%) is the percentage of immobile copepods compared to the total number of copepods that were exposed to a certain concentration of test substance (= 20). Based on these values, a two-parameter log-logistic curve was fitted using the 'drm' function of the R software 'drc' package to describe the relationship between the immobility (%) after 96 h of exposure and the concentration of test substance (Ritz et al., 2015). From this curve, the EC_{10} at 96 h was estimated using the 'ED' function of the R software 'drc' package, i.e., the concentration of test substance for which 10 % of the exposed *N. spinipes* adults are immobilized (Ritz et al., 2015). A 95 % confidence interval was calculated for the EC_{10} values, using the delta method. We chose to calculate the EC_{10} values for comparison of leachates treatments because an immobility of 50 % was never reached during the tests, making it too difficult to estimate the EC_{50}. For the exposure to the reference substance $K_2Cr_2O_7$ the EC_{50} at 96 h was calculated (i.e., the concentration of $K_2Cr_2O_7$ which immobilizes 50 % of exposed copepods). This value was compared with the mean EC_{50} after 96 h of exposure to $K_2Cr_2O_7$ at 7 PSU and 20 ± 2 °C, given in Annex C of the ISO 14669 protocol (ISO, 1999). This comparison was used to evaluate the repeatability of the test.

4. RESULTS

4.1 Algal growth inhibition tests

4.1.1 Quality control test

The mean specific growth rates of the $K_2Cr_2O_7$ treatments for the quality control test of ISO protocol 10253 and standard deviations were equal to: 0.97 ± 0.17, 0.94 ± 0.10, 0.93 ± 0.03, 0.68 ± 0.19, 0.38 ± 0.11, -0.08 ± 0.06 d^{-1} for treatments with 0, 2.5, 5, 10, 20, and 40 mg / L respectively (Subsection 3.3.2) (ISO, 2016a). The null hypothesis of a one-way ANOVA test, which assumes there are no different means between groups, was rejected ($p = 1.86 \cdot 10^{-7}$). Post-hoc comparisons (Dunnett test) showed that the mean specific growth rate of algae exposed to $K_2Cr_2O_7$ concentrations of 10, 20 and 40 mg / L were significantly different from the mean specific growth rate of the algae in the control flasks ($p = 0.038$, $p = 1.4 \cdot 10^{-4}$ and $p = 1.2 \cdot 10^{-8}$, respectively) (Figure 4.1a). The EC_{50} after 72 h and its 95 % confidence interval were equal to 11 [3 : 18] mg / L. This was based on the calculated mean growth inhibitions (%) of the test vessels per treatment, with the exception of the treatment with 40 mg / L, and the fitted four-parameter log-logistic curve (Figure 4.1b).



(a) (b)

Figure 4.1: Algal growth inhibition 72 h quality control test with potassium dichromate ($K_2Cr_2O_7$) concentrations (mg / L), using three replicates for concentrations of $K_2Cr_2O_7$ greater than zero and six replicates for the controls with 0 mg / L $K_2Cr_2O_7$ (a) Boxplots of specific growth rates (d^{-1}) of *P. tricornutum* versus $K_2Cr_2O_7$ concentrations of 0, 2.5, 5, 10, 20, and 40 mg / L. The specific growth rates were calculated with Equation 3.1 using the measured cell densities (cells / mL) at the start and end of the 72 h exposure to $K_2Cr_2O_7$ *(* = significantly different mean specific growth rates from the control, $p < 0.05$)*. (b) Fitted four-parameter log-logistic dose-response curve *(blue line)* for the calculated mean and standard deviations *(black dots and error bars)* of algal growth inhibition (%) after 72 h exposure to $K_2Cr_2O_7$ concentrations of 0, 2.5, 5, 10, and 20 mg / L. The highest concentration of 40 mg / L was not included because the specific growth rate at this concentration was negative. The resulting EC_{50} and its 95 % confidence interval were equal to 11 [3 : 18] mg / L.

4.1.2 Ecotoxicity of pristine PVC leachates

The first leachates for which the ecotoxicity was assessed were made with pristine PVC. After a 72 h exposure of *P. tricornutum* to 90 % v / v pristine PVC leachates (treatment C1) and a control (0 % v / v, treatment C0), the mean specific growth rates and standard deviations were equal to: 0.95 ± 0.06 and 1.08 ± 0.06 d^{-1} for C1 and C0 respectively. The null hypothesis of a one-way ANOVA, which states that all mean specific growth rates are equal, was rejected ($p = 0.011 < 0.05$) (Figure 4.2).

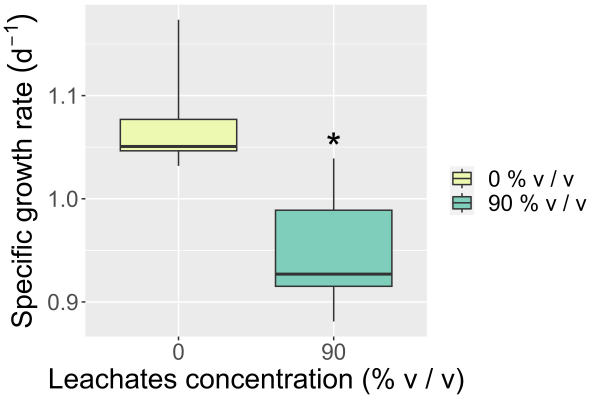


Figure 4.2: Boxplots of specific growth rates (d^{-1}) of *P. tricornutum* after 72 h exposure to control algal growth medium (leahates concentration = 0 % v / v) and pristine PVC leachates solution (leachates concentration = 90 % v / v), using five replicates for each concentration *(* = significantly different mean specific growth rate from the control, $p < 0.05$)*.

4.1.3 Ecotoxicity of PLA leachates

The specific growth rates (d^{-1}) of *P. tricornutum* after 72 h of exposure to 0, 10, 24, and 60 % v / v leachates concentrations (treatments C0, C1, C2, and C3 respectively) of each PLA plastic were calculated (Figure 4.3). The means and standard deviations of these values are given in Table 4.1. Only the one-way ANOVA with seawater-aged SR-PLA showed a significant difference between the mean specific growth rates at different leachates concentrations (p = 0.001). Based on a Dunnett test it was observed that all three seawater-aged SR-PLA concentrations result in significantly different mean specific growth rates than the control (p = 0.004 for C1 - C0, p = 0.03 for C2 - C0, p = 0.001 for C3 - C0).

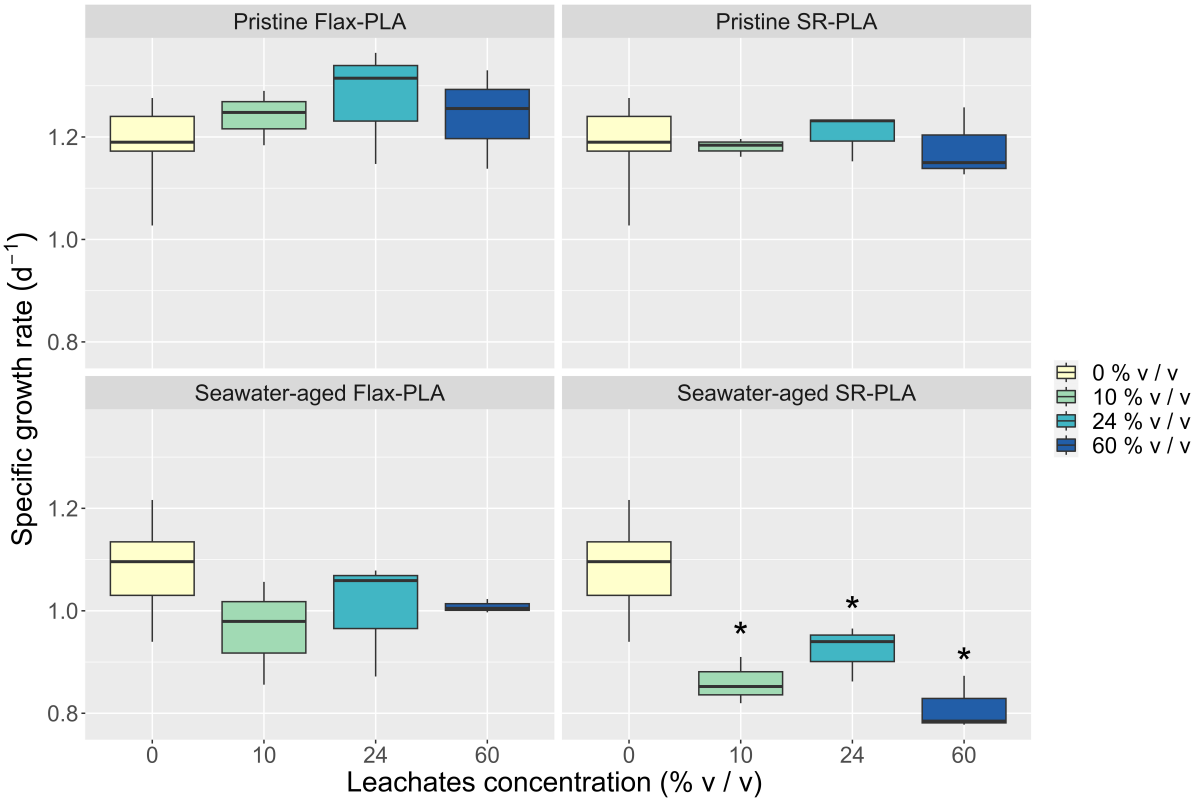


Figure 4.3: Boxplots of the specific growth rate (d^{-1}) of *P. tricornutum* after 72 h exposure to dilution series (60, 24, and 10 % v / v) of pristine SR-PLA, seawater-aged SR-PLA, pristine Flax-PLA and seawater-aged Flax-PLA leachates, using three replicates each, and to control medium (0 % v / v plastic leachates), using six replicates per batch (one pristine and one seawater-aged) *(* = significantly different mean specific growth rates from the control, $p < 0.05$)*.

Table 4.1: Mean specific growth rates (d^{-1}) of *P. tricornutum* after 72 h exposure to control medium (0 % v / v plastic leachates) and to dilution series (60, 24, and 10 % v / v) of pristine SR-PLA, pristine Flax-PLA, seawater-aged SR-PLA, and seawater-aged Flax-PLA leachates *(blue = significantly different mean specific growth rates from the control, $p < 0.05$)*.

Leachates solution		
Polymer	**Concentration (% v / v)**	**Mean specific growth rate after 72 h ± standard deviation (d^{-1})**
pristine batch	0 (control)	1.18 ± 0.09
seawater-aged batch	0 (control)	1.08 ± 0.10
pristine SR-PLA	10	1.18 ± 0.02
pristine SR-PLA	24	1.21 ± 0.05
pristine SR-PLA	60	1.18 ± 0.07
pristine Flax-PLA	10	1.24 ± 0.05
pristine Flax-PLA	24	1.28 ± 0.11
pristine Flax-PLA	60	1.24 ± 0.10
seawater-aged SR-PLA	10	0.86 ± 0.05
seawater-aged SR-PLA	24	0.92 ± 0.05
seawater-aged SR-PLA	60	0.81 ± 0.05
seawater-aged Flax-PLA	10	0.96 ± 0.10
seawater-aged Flax-PLA	24	1.00 ± 0.11
seawater-aged Flax-PLA	60	1.01 ± 0.01

4.2 Copepod acute lethal toxicity tests

4.2.1 Ecotoxicology of pristine and environmental PVC leachates at 22 °C and 25 °C

The number of immobile *N. spinipes* was evaluated in each well after 96 h of exposure to pristine and environmental PVC leachates dilution series at 22 °C and 25 °C (Table 4.2). Two-parameter log-logistic functions were fitted based on the immobility (%) and the corresponding leachates concentration (% v / v) causing this immobility (Figure 4.4). From the fitted curves, EC_{10} estimates with standard deviations and 95 % confidence intervals of 40.74 ± 24.90 % v / v [-28.40 : 109.88] and 16.01 ± 9.46 % v / v [-10.25 : 42.27] were observed for 96 h of pristine PVC leachates exposure at 22 and 25 °C respectively. For environmental PVC the EC_{10} estimates with standard deviations and 95 % confidence intervals were equal to 25.31 ± 16.00 % v / v [-19.09 : 69.71] and 8.93 ± 7.84 % v / v [-12.83 : 30.69] for 96 h of exposure at 22 and 25 °C respectively. When comparing the findings between the two temperatures, it is found that, for both pristine and environmental PVC, the 96-h EC_{10} is not lower when exposure takes place at a 3 °C higher temperature, considering the 95 % confidence intervals.

Table 4.2: Results after 96 hours of the copepod acute lethal toxicity tests, where *N. spinipes* were exposed to controls (0 % v / v) and dilution series of 100.0, 75.00, 56.25, 42.19, and 21.09 % v / v pristine PVC leachates solutions at two temperatures: 22 °C and 25 °C. T is the temperature (°C), N is the number of surviving copepods at each concentration and P is the percentage of immobility of the copepods at each concentration.

T (°C)	Leachates solution		N	P (%)
	Polymer	Concentration (% v / v)		
22	pristine PVC	0 (control)	20	0
22	pristine PVC	21.09	18	10
22	pristine PVC	42.19	20	0
22	pristine PVC	56.25	15	25
22	pristine PVC	75.00	18	10
22	pristine PVC	100.0	15	25
25	pristine PVC	0 (control)	19	5
25	pristine PVC	21.09	18	10
25	pristine PVC	42.19	16	20
25	pristine PVC	56.25	11	45
25	pristine PVC	75.00	14	30
25	pristine PVC	100.0	11	45
22	environmental PVC	0 (control)	19	5
22	environmental PVC	21.09	18	10
22	environmental PVC	42.19	18	10
22	environmental PVC	56.25	16	20
22	environmental PVC	75.00	10	50
22	environmental PVC	100.0	14	30
25	environmental PVC	0 (control)	18	10
25	environmental PVC	21.09	18	10
25	environmental PVC	42.19	11	45
25	environmental PVC	56.25	13	35
25	environmental PVC	75.00	11	45
25	environmental PVC	100.0	11	45

4.2.2 Quality control test

For the quality control test, the EC_{50} value with standard deviation and 95 % confidence interval for 96 h of copepod exposure to the reference substance potassium dichromate ($K_2Cr_2O_7$) at 22 °C was estimated to be equal to 8 ± 1.58 mg / L [4 : 12].

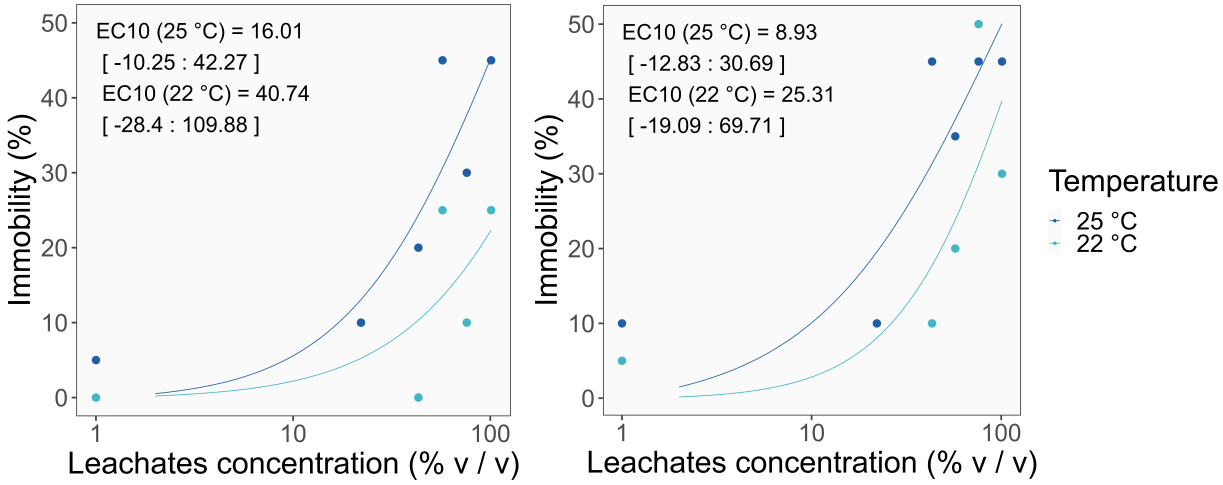


Figure 4.4: Fitted curve of the immobility (%) of *N. spinipes* caused by (a) pristine PVC leachates exposure and (b) environmental PVC leachates exposure. The curves were fitted based on acute lethal toxicity tests with controls (0 % v / v) and dilution series of 100.0, 75.00, 56.25, 42.19, and 21.09 % v / v PVC leachates solutions at two temperatures: 22 ˚C and 25 ˚C. The EC_{10} (% v / v) estimates and 95 % confidence intervals from the fitted curves are given for each temperature.

5. DISCUSSION

Our study indicates that leachates of pristine PVC and seawater-aged SR-PLA plastics induced ecotoxicological effects on *P. tricornutum* and that there are no significant differences in induced ecotoxicological effects of pristine and environmental PVC leachates on *N. spinipes* when including a 3 °C temperature rise as an additional stressor. From the assessments of the effects of plastic leachates on *P. tricornutum*, it was found that pristine PVC and seawater-aged SR-PLA leachates reduced algal growth rates after 72 h, while seawater aged Flax-PLA, pristine Flax-PLA and pristine SR-PLA did not. The combined effects of a 3 °C temperature rise and plastic leachates on *N. spinipes* did not result in significantly different effective concentrations of PVC leachates to immobilize the copepods at different temperatures. This differed from the expected enhancement of acute lethal toxicity by an increase in temperature as a potential consequence of higher energy requirements for increased metabolic rates.

5.1 Quality controls for ecotoxicological tests

The 72-h quality control test with the reference substance potassium dichromate ($K_2Cr_2O_7$) that was done to verify the comparability and reliability of our algal growth inhibition tests resulted in a 95 % confidence interval for the EC_{50} (= [3 : 18] for 11 mg / L) that included the EC_{50} value given in Annex B of the ISO protocol 10253 (16.21 ± 1.72 mg / L) (ISO, 2016a). The EC_{50} estimate for our 96 h copepod acute lethal toxicity quality control test with $K_2Cr_2O_7$ (= 8 mg / L, 95 % confidence range [4 : 12]) was lower than the mean EC_{50} of 18.7 mg / L mentioned in Annex C of ISO protocol 14669 (ISO, 1999). However, the absolute standard deviation for reproducibility mentioned in the protocol is equal to 7.85 mg / L. We decided to accept the copepod acute lethal toxicity test method based on following reasons: 1) the upper limit of our 95 % confidence interval still lies within the reproducibility range of ISO protocol 14669 (ISO, 1999), 2) the results in the protocol originate from only 11 different laboratories and already show relatively high standard deviations in comparison with the mean EC_{50}, and 3) it was observed that the controls did not have immobility percentages greater than 10 % (Table 4.2) (ISO, 1999). The implementation of a rigorous quality control test with a known reference substance serves as a fundamental cornerstone in environmental toxicity assessments because it validates the precision and credibility of the test systems.

The ISO protocols also contain reporting guidelines for researchers, which are important to reach reproducibility and comparability in plastic pollution research (Cowger et al., 2020). One of the reporting requirements of ISO protocol 10253 is to make sure that the control pH did not increase more than 1.0 during the quality control test (Appendix C.1.1). This extra validation is needed to exclude the possibility that variations in pH during the tests might have influenced the results (ISO, 2016a). For our study, we can thus state that the procedure and sensitivity of the 72 h algal growth inhibition test and 96 h copepod acute lethal toxicity test were following the standardized ISO protocols 10253 and 14669 respectively (ISO, 2016a, 1999).

5.2 Effects of plastic leachates

Standardized algal growth inhibition tests were set up with both fuel-based and bio-based plastic leachates to assess their ecotoxicity (ISO, 2016a). In this research, a pristine PVC leachates solution at a concentration of 90 % v / v (Figure 4.2) and seawater-aged SR-PLA leachates solutions at concentrations of 60, 24, and 10 % v / v (Figure 4.3), that were all made from 80 g / L plastic stock solutions, showed significant inhibition of *P. tricornutum* growth. The leachates stock solutions with a solid-to-liquid ratio of 80 g / L were made in accordance with Capolupo et al. (2020) and Lithner et al. (2009), because of the importance that the research field on plastic pollution works towards comparable results (Cowger et al., 2020).

5.2.1 Ecotoxicity of pristine PVC leachates

Since the mean specific growth rate (d^{-1}) for *P. tricornutum* species exposed to the pristine PVC leachates solution for 72 h was significantly lower than the mean specific growth rate for the diatoms in the control flasks ($p < 0.05$) (Figure 4.2), we evaluate that the concentration of 90 % v / v leachates solution from 80 g / L pristine PVC has a toxic effect on the marine diatom species. With a similar test, Capolupo et al. (2020) found a 72-h EC_{50} equal to 35 % v / v of 80 g / L PVC leachates, considering the specific growth rate of the marine algae *Skeletonema costatum*. Tetu et al. (2019) found that higher PVC leachates concentrations resulted in greater population density reductions of bacteria of the cyanobacterial genus *Prochlorococcus*. Although these are findings for other marine primary producers, it gives an indication of the ecotoxicity of our PVC leachates. Since the tested concentration is well above the 72-h EC_{50} value calculated by Capolupo et al. (2020) and the leachate exposure alters cell populations in a dose-dependent way (Tetu et al., 2019), it was expected that 72 h of exposure to a concentration of 90 % v / v pristine PVC leachates would have a toxic effect on our algae, resulting in a reduced mean specific growth rate. Multiple researchers hypothesize that the toxicity of PVC results from endocrine-disruptive phthalate molecules (Lithner et al., 2009; Oliviero

et al., 2019; Sree et al., 2023). Phthalates are widely used in PVC and can easily leach out of the polymer matrix since their goal as plasticizers is to reduce the chemical affinity between molecules (Hermabessiere et al., 2017; Oehlmann et al., 2009). However, phthalates are not the only components of PVC leachates that are capable of inducing toxic effects. Of all plastic materials, PVC needs the most additives, many of which are known to be toxic, including heavy metals, flame retardants, and bisphenol A (Baršienė et al., 2006; Bollmann et al., 2012; Canesi and Fabbri, 2015; Murphy, 2001; Simon et al., 2021). We are unsure if the toxic effect of PVC leachates on *P. tricornutum* was caused by a single component or by a combination of substances. To get further insights it is advisable to do chemical analyses of the leachates, via gas chromatography-mass spectrometry for example (Capolupo et al., 2020).

5.2.2 Ecotoxicity of PLA leachates

For the plastic leachates solutions from seawater-aged SR-PLA, all three concentrations of 60, 24, and 10 % v / v showed significant acute toxicity for the marine diatom after 72 h of exposure (Figure 4.3). The same concentrations and exposure duration of pristine SR-PLA, pristine Flax-PLA and seawater-aged Flax-PLA leachates did not show significant toxic effects on the growth of the model species. This indicates that only the SR-PLA plastics produced toxic leachates after a seawater immersion of two months. Zimmermann et al. (2020) and Uribe-Echeverría and Beiras (2022) studied the chemical toxicity of multiple plastics, including PLA, to, respectively, *Daphnia magna* and *Paracentrotus lividus* larvae fertilization and development. Both studies found that the PLA leachates were ineffective in inducing toxicity for the species, as for our two pristine PLA leachates and seawater-aged Flax-PLA leachates. Interestingly, Zimmermann et al. (2020) suggest that the chemical toxicity is specific to the individual material and not necessarily to a polymer type. Additionally, taking both MPs and leachates of PLA into account, they conclude that bio-based and/or biodegradable plastics may cause toxicity similar to conventional fuel-based polymers. Recently, Capolupo et al. (2023) assessed effects of PLA leachates on *Mytilus galloprovincialis*. They found that the PLA leachates significantly impaired the physiological larvae development and motility, while survival was not affected. Our results show that leachates from seawater-aged SR-PLA are more toxic than those from pristine SR-PLA, which is in accordance with Weber et al. (2020) and Bejgarn et al. (2015) who found that toxicity of plastic leachates varied as a function of weathering and polymer type. These current findings thus give an indication that the toxicity we found for seawater-aged SR-PLA is specific to our endpoint, weathering technique, and reinforcement components. This underlines the importance of considering the individual material's characteristics, its leachates composition, and potential leachates toxicity, rather than relying solely on broad polymer categories (Gunaalan et al., 2020). While bio-based plastics are often con-

sidered environmentally friendly alternatives for fuel-based plastics (Iwata, 2015), our findings highlight the necessity of rigorous assessment to ensure they align with their intended eco-friendly properties. It is important to include assessments of the complex mixtures of additives because it is not guaranteed that, if individual components are non-toxic, the mixture of these components will be harmless as well (Curto et al., 2021). Therefore, using similar toxicity tests becomes a valuable tool in distinguishing plastics that might or might not pose risks to marine ecosystems, thereby discerning which materials are more suitable for marine deployment, potentially contributing to more sustainable choices in plastic production and usage.

5.3 Temperature effects on ecotoxicity of plastic leachates

5.3.1 Ecotoxicology of pristine and environmental PVC leachates at 22 °C and 25 °C

A 3 °C temperature rise did not result in significantly enhanced copepod immobility when (separately) exposed to two types of PVC leachates (Figure 4.4). After exposing *N. spinipes* to a dilution series of pristine and environmental PVC leachates for 96 h at 22 °C and 25 °C, no significant differences in immobility were observed between leachates concentrations nor between temperatures (Figure 4.4). Interestingly, this implies that the toxic effects of both pristine and environmental PVC leachates on *N. spinipes* adults remain unaltered even in the face of the 3 °C temperature increase that is expected due to ocean warming by 2100 according to RCP8.5 (i.e., "bussiness as usual") of the IPCC (Abram et al., 2019; Bindoff et al., 2019). However, we should take into account the exact temperatures that were used for this assessment: 22 °C and 25 °C. Heugens et al. (2001) 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 this experiment, it is possible that the 25 °C temperature does not reach this limit since *N. spinipes* species are known for their ability to live in a broad temperature range (Bengtsson, 1978). Caution is thus needed when trying to compare these results to temperature effects on the toxicity of leachates for other species.

The 96 h effective concentrations EC_{10} indicate the substance concentration at which 10% of the maximum effect (i.e. immobility of all copepods) is produced. The risk of toxicity of leachates to the test organisms is low when the EC_{10} value is higher than the concentration measured in the environment. Often EC_{50} values (i.e. 50% of the maximum effect) are used to compare toxicities of substances, but we chose to calculate the EC_{10} because our highest PVC leachates concentrations (100 % v / v from 80 g / L PVC) did not affect the mobility of more than 50% of the

copepods, which means our estimations for the EC_{50} values would be more uncertain (Noel et al., 2018). (Capolupo et al., 2020) found EC_{50} of PVC leachates equal to 34.6%, 16.45%, and 3.01% when considering *Skeletonema costatum* growth inhibition, *Mytilus galloprovincialis* gamete fertilization, and *Mytilus galloprovincialis* embryo-larval development respectively, with leachates that were made from 80 g / L PVC. Their low value for the EC_{50} for *M. galloprovincialis* embryo-larval development showed that although in the natural environment leachates will be subjected to dilution, high plastic accumulation zones are potentially influenced by the leaching of additives. Obtaining effective concentration values is thus essential to determine the toxicity of leachates in the environment.

5.3.2 Future possibilities

This research opens up the opportunity to perform tests considering the effects of temperature rise on the ecotoxicity of plastic leachates for the whole life cycle of *N. spinipes*. In future studies, we can uncover potential life cycle stage-specific susceptibilities by evaluating the impact of temperature elevation on plastic leachates' toxicities at various life stages, including larvae. Conducting larval development tests on *N. spinipes* to assess the ecotoxicity of leachates can be done in a standardized way with ISO protocol 18220 (ISO, 2016b). When larval development or survival is affected due to exposure to leachates and/or elevated temperatures, it can result in reduced recruitment of new individuals into populations and can disrupt the balance of species within the ecosystem (Marshall and Keough, 2006). Such changes can ripple through the food web, impacting predators, prey, and competitors. Shifting the focus to larvae could thus provide insights into differential sensitivities and highlight important windows of vulnerability, enhancing our understanding of the larger ecological consequences of plastic pollution on this species, and potentially opening perspective for population shifts (Marshall and Keough, 2006).

Additional to leachates' effects on multiple life stages, this research team aims to assess the impacts of MPs and climate change-induced stressors on aquatic species, thereby including multiple facets of the growing plastic pollution problem in an environmentally relevant context. Appendix B includes further method developments for MPs risk assessments. Eventually, including MPs and NPs in leachates mixtures when assessing the ecotoxicity is interesting as well, especially when investigating leachates of weathered plastics as they will release MPs and NPs in the environment during the weathering process. Using such complex mixtures (assuming environmentally realistic MPs and NPs concentrations) reflects more realistic conditions, which is interesting to perform relevant risk assessments of plastic pollution, but it also makes the interpretation of the responses of exposed organisms more complex, because physical and chemical effects will then be harder to distinguish (Koel-

mans et al., 2022). To make ecotoxicology tests even more realistic, more climate-change-related stressors can be included in risk assessments of plastic products, such as ocean heatwaves, ocean acidification, and ocean deoxygenation (Cooley et al., 2022), anticipating future conditions.

6. CONCLUSION

Our study delved into the ecotoxicological effects of plastic leachates on two abundant species at the base of the food chain and their potential interactions with temperature stress. The assessment of plastic leachates effects on *Phaeodactylum tricornutum* revealed that pristine PVC and seawater-aged SR-PLA leachates led to reduced algal growth rates, suggesting a notable impact on this primary producer. However, the absence of significant sublethal toxic effects in tests with other types of leachates, such as pristine Flax-PLA and seawater-aged Flax-PLA, highlights the specificity of toxicity responses to particular plastic materials and their degradation states. Furthermore, our study sheds light on the complex dynamics between plastic leachates and temperature, revealing that a 3 °C rise, representing ocean warming by 2100, did not magnify the toxic impact of pristine and environmental PVC leachates on adults of the harpacticoid copepod species *Nitokra spinipes*. While our findings contribute valuable insights, they also underline the need for tailored assessments considering diverse plastic materials, leachates compositions, and life cycle stages. We advocate for a holistic approach in future studies, encompassing a variety of plastic types, concentrations, and biological interactions using comparable and reproducible ecotoxicological assessment methods to unravel the intricate mechanisms underlying plastic pollution's ecological implications.

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APPENDIX A

SUPPORTING PRACTICES

A.1 Determining algal cell densities

A.1.1 Cell density of *P. tricornutum*

To practice the determination of the cell density of the *P. tricornutum* cultures, three 1 mL dilutions (1/5, 1/25 and 1/125) were made from 1 mL of a culture with filtered artificial seawater (Section 3.1.1). Subsequently, 10 μL of the different dilutions was injected in both counting chambers of a hemocytometer, as described by ChemoMetec. The morphology of *P. tricornutum* cells was then studied under a light microscope (DM1000, Leica) with 10x objective lens to be able to count the correct cells. Both counting the cells directly from the microscope with a lab-count denominator and indirectly from pictures with the image processing software ImageJ were practiced multiple times. Once the number of cells was counted, the cell density could be calculated for both methods, taking into account the volume that was in the hemocytometer squares and picture frames respectively.

A.1.2 Cell density of *R. salina*

For the further method developments in Appendix B, *R. salina* cell densities were determined using a FlowCam with a 4x magnification objective at a flow rate of 1.700 mL / min. Before each measurement, the FlowCam was cleaned with 70 % ethanol and milli-Q water at a flow rate of 3.000 mL / min.

A.2 Algal growth rate trial tests

A.2.1 Specific growth rate for a new *P. tricornutum* culture

As a first trial test, the growth rate of a newly made *P. tricornutum* culture was studied. The cell density (cells / mL) of this culture was measured every 24 ± 2 h for a duration of four days, and once more after seven days. After log-transferring the cell densities, Figure A.1 was obtained. The specific growth rate (d^{-1}) is calculated as the slope between two time points.

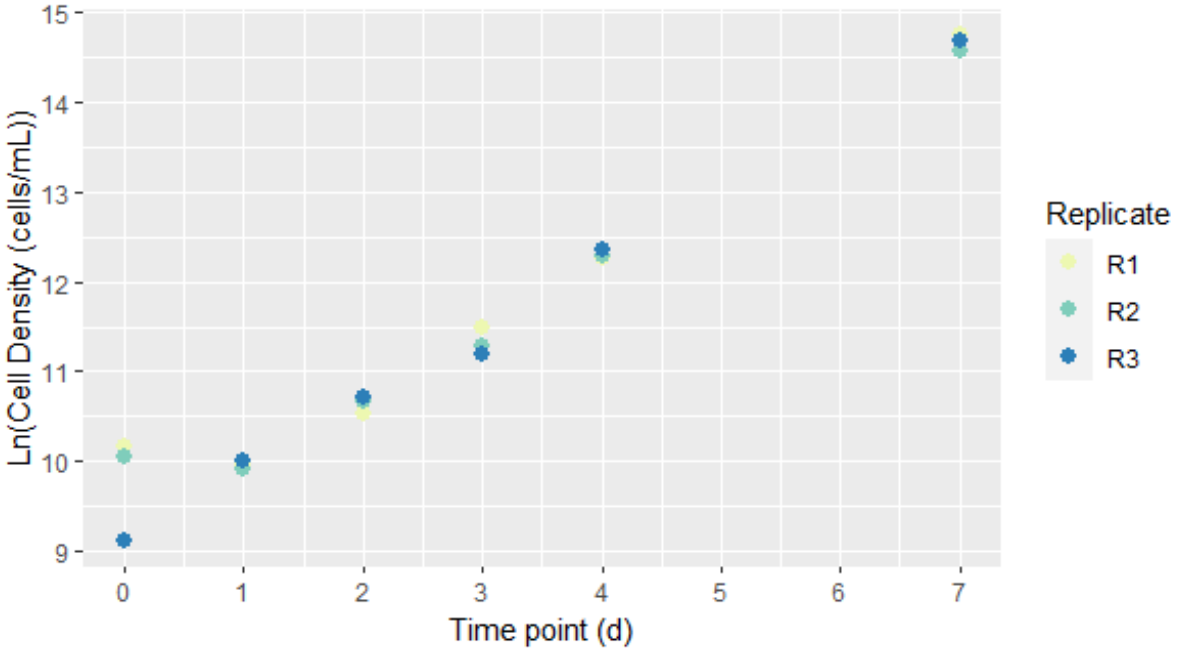

Figure A.1: Scatterplot of log-transferred *P. tricornutum* cell densities (cells / mL) in time (d), starting from a new culture.

This trial experiment showed that there is a lag phase in the growth rate of a new *P. tricornutum* culture before reaching an exponential growth rate. Aim was to emphasize the use of pre-cultures before starting an algal growth inhibition test.

A.2.2 Specific growth rate for a *P. tricornutum* pre-culture

A second trial test on the specific growth rate of *P. tricornutum* was done following the ISO 10253 protocol (ISO, 2016a). For this test, a pre-culture was made four days prior. This pre-culture was used to make six control cultures (pre-culture + growth medium). The cell densities (cells / mL) of these cultures were determined every 24 ±2 h for a duration of 72 h. This data was again log-transferred to visualize the specific growth rate (d^{-1}) (Figure A.2).

Since a linear growth curve indicates exponential growth, whereas a leveling off indicates that cultures have entered the stationary phase, it was confirmed that the pre-culture was growing at an exponential rate during 72 h.

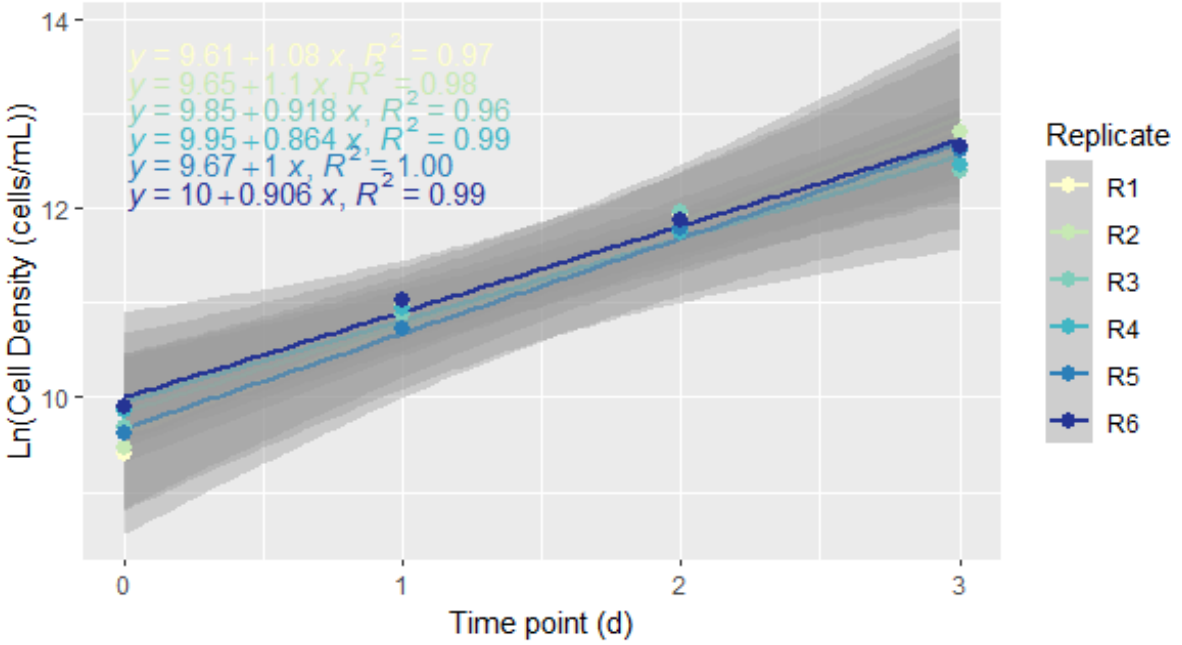

Figure A.2: Scatterplot with linear regression lines of log-transferred *P. tricornutum* cell densities (cells / mL) in time (d), starting from a four-days-old pre-culture.

A.3 Identifying and transferring copepods

A.3.1 *Nitokra spinipes* in diluted natural seawater

The identification of the different *N. spinipes* life cycle stages was studied under a Leica S8 APO stereomicroscope. Transferring them was then practiced under the same microscope. A 100 μL Eppendorf pipette was used to transfer the copepods along with 10 μL of DNSW to different wells filled with 40 μL diluted natural seawater and 40 μL 70 % ethanol. After one week each specimen was checked again to study their appearance along with their fecal pellets.

A.3.2 *Nitokra spinipes* in sediments

The retrieval of copepods from sediments was practiced. A 180 μm mesh that stops most of the sediment was put on top of a 60 μm mesh. By rinsing the 60 μm mesh upside down, the copepods on top of the mesh were successfully retrieved. Additionally, sediment maintenance was done, by removing the supernatant DNSW with a syringe and replacing it with new brackish water, keeping the culture vibrant. These copepods were not used in the experiments mentioned in this thesis, but will be used in future experiments where the copepods will be exposed to MPs in sediment, instead of in the water column (Appendix B).

APPENDIX B

FURTHER METHOD DEVELOPMENT

B.1 Objectives

Further method development was done to assess the effects of MPs on *N. spinipes*. As a first step, the ingestion of PS and PLGA beads was assessed. It was expected that the copepod species would ingest MPs since they are filter feeders. Ingestion of MPs could have metabolic and/or energetic costs. For this reason, the oxygen consumption rate and filtration rate of *N. spinipes* were studied after exposure to PLGA beads. Overall this method development aims to contribute to the growing body of knowledge on the impacts of plastic pollution on marine organisms.

B.2 Microplastic beads stock solutions

Fluorescent microspheres with an average diameter of 5 μm and 10 μm of PS and 5 μm of PLGA were purchased from Phosphorex, LLC. Stock solutions with PS and PLGA were prepared from the purchased beads to perform exposure tests. For each stock solution, MPs beads were dispersed in test vials with 1 mL of 0.1 % v / v Tween-80 (i.e., a nonionic surfactant to facilitate suspension) (Cospheric LLC) and 1 mL of Milli-Q water, followed by five minutes of vortexing. This resulted in MP stock suspensions of $1.25 \cdot 10^9$ particles / mL. The particle concentration was checked with a hemocytometer under a fluorescent microscope with 10x objective lens (EL6000, Leica Microsystems). From this, six 2 mL tubes (three per stock solution) were prepared with concentrations of $7 \cdot 10^4$, $1 \cdot 10^4$, and $1 \cdot 10^3$ particles / mL.

B.3 Maintenance of model species

The model species *Nitokra spinipes* were maintained in the same way as described in Section 3.2. Three weeks before MPs beads exposure experiments, the *N. spinipes* food source was changed to *Rhodomonas salina* suspension. The amount of *R. salina* added resulted in a concentration of algae in the dishes greater than $2.5 \cdot 10^5$

cells / mL, as suggested by ISO protocol 18220 (ISO, 2016b). *R. salina* were cultured in 200 mL of L1 culture medium (25 PSU) (Guillard and Hargraves, 1993). This was done in pre-sterilized 500 mL Erlenmeyer flasks. The cultures were kept at 18 ± 2 °C and were continuously renewed. To avoid salinity changes in the *N. spinipes* cultures when feeding them with *R. salina*, algae suspensions of 7 PSU were made. This was established by centrifuging four 50 mL tubes with *R. salina* culture twice at 1,500 rpm for 5 min. After each centrifugation, the supernatant was discarded and a total of 10 mL 7 PSU DNSW (Subsection 3.1.1) was added to the algae cells.

B.4 Copepod microplastic ingestion tests

B.4.1 Preparation of the PS beads exposure test: gut clearance

Eight wells on a 24-well plate were filled with 2 mL of DNSW (Subsection 3.1.1) and five *N. spinipes* adults and kept in dark at 22 ± 1 °C for 30 h. During this time, gut clearance took place. The fecal pellets were removed from the wells every few hours and the amount of copepods in the wells was checked to make sure no copepods were removed in the process.

B.4.2 PS beads exposure

To check which MPs beads tests would be feasible to perform exposure tests on *N. spinipes* species, it was examined if they ingest 5 μm and/or 10 μm PS microspheres. After thirty hours of gut clearance, all selected *N. spinipes* species were exposed to about $1 \cdot 10^4$ particles / mL of 5 μm PS and 10 μm PS each (Appendix B.2), while being fed with about $1 \cdot 10^5$ cells / mL *R. salina*. Ten copepods were then removed from the wells after 1, 3, 18, and 24 h and put in 4 % formalin. These copepods were studied under a fluorescent microscope with a 10x objective lens (EL6000, Leica Microsystems). The number of copepods that ingested PS, as well as the positioning of these particles was determined.

B.4.3 PS beads ingested by *N. spinipes*

The percentage of *N. spinipes* that ingested MPs per time point (1, 3, 18, and 24h) was observed (Figure B.1). After 18 h, 80 % of the copepods had ingested PS beads.

This experiment confirms that our model species ingest MPs when we do exposure experiments. This is as expected for filter feeders, meaning they consume particles suspended in water. Mueller et al. (2020) used similar sizes of PS beads (0.1 - 10.0 μm) and found that the effects of PS beads on nematodes correlate with the

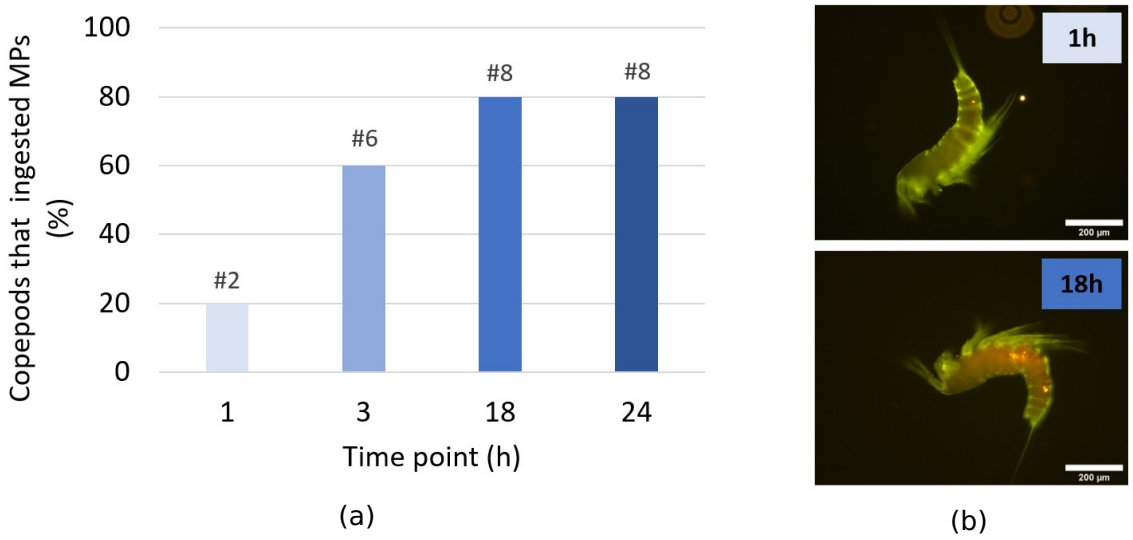

Figure B.1: (a) Barplot representing the percentage of copepods that ingested MPs (%) after 1, 3, 18, and 24 h of exposure to $1 \cdot 10^4$ particles / mL of 5 μm PS and 10 μm PS each, while being fed with about $1 \cdot 10^5$ cells / mL *R. salina*. The absolute number of copepods that ingested these particles is given on top of the bars. (b) Pictures of *N. spinipes* viewed under a fluorescent microscope with 10x objective lens (EL6000, Leica Microsystems) with a blue-light filter after 1 and 18 h of exposure to PS beads *(white scale bar = 200 µm)*.

total surface area of the beads per volume, while they were independent of the size of the beads. Both sizes of PS beads could thus be further investigated, but we expected exposures with 5 μm beads would be more successful as they are similar to the size of the prey species *R. salina*. It could be interesting to study the elimination of MPs in the future, as done by Wang et al. (2019), to estimate the time that MPs beads were inside of our test species, giving us further insights into findings from exposure experiments. After ingestion, different internal effects can be studied such as metabolic and energetic costs due to physical ingestion. Another concern with MPs is the potential release of leachates in the digestive tract that could potentially have toxic effects on organisms that ingest the MPs (Catarino et al., 2021; Khan et al., 2022). Questions remain about whether investigated effects of MPs are solely physical or if they are partly because of the release of chemicals by these MPs (Curto et al., 2021). Understanding how MPs are ingested and potentially affect the organisms is crucial for assessing the impact of plastic pollution on marine ecosystems.

B.4.4 PLGA beads exposure

Considering bio-based polymers, the copepods were exposed to three different concentrations of 5 μm PLGA beads: $1{\cdot}10^3$, $1{\cdot}10^4$, and $7{\cdot}10^4$ particles / L (Appendix B.2). For this exposure experiment, we assessed the ingestion of the beads after 48 h of exposure.

B.4.5 PLGA beads ingested by *N. spinipes*

After 48 h of exposing *N. spinipes* to $1{\cdot}10^3$, $1{\cdot}10^4$, and $7{\cdot}10^4$ particles / L of 5 μm PLGA beads, ingestion of the plastic particles had taken place for all three exposure concentrations. These findings led to trial experiments considering possible metabolic and energetic costs due to PLGA ingestion in *N. spinipes* (Appendix B.5).

B.5 Trial experiments to assess energetic and metabolic costs of MPs

Multiple trial experiments were performed with 5 μm PLGA beads to assess energetic and metabolic costs of MPs in *N. spinipes*. The oxygen consumption rate represents the metabolic energy level of the organisms, while the filtration rate represents the feeding behavior specifically. Questions that are interesting to answer in the future are: (1) Are organisms experiencing physiological stress or changes in energy expenditure due to microplastic exposure? (2) How do MPs affect an organism's ability to efficiently filter and capture food particles? (3) How might changes in the metabolic and filtration rates of key organisms affect nutrient cycling, energy flow, and overall ecosystem health? (4) Are certain species more susceptible to the effects of microplastics based on their feeding strategies and metabolic demands? (5) Are certain plastics more harmful and can we contribute to decision-making for plastic production and consumption legislation? (6) What are the potential long-term implications of altered metabolic and filtration rates on individual organisms, populations, and ecosystem dynamics? To answer these questions, alignment methods will be crucial for interstudy comparison (Koelmans et al., 2022). We illustrate our first steps to contributing to these method developments.

B.5.1 Copepod oxygen consumption rate tests

To perform oxygen consumption rate tests, oxygen sensor spots and an OXY 4 SMA (G3) measuring device from the company PreSens Precision Sensing GmbH were used. At first, oxygen sensor spot integration onto glass vials was practiced. These vials were dried and used to perform trial experiments.

To prepare the oxygen consumption rate test, twenty *N. spinipes* and 10 μL of $1{\cdot}10^3$, $1{\cdot}10^4$, and $7{\cdot}10^4$ particles / L 5 μm PLGA were added (separately) to 5 mL of DNSW with algal feed on 6-well plates, resulting in three different PLGA treatments (C2, C3 and C4). Additionally, one starvation treatment (C1) where copepods were put in DNSW only and one control treatment (C0) with DNSW and algal feed were made. After 24 h of exposure to the different treatments, five copepods were added to

10 mL glass vials with oxygen sensor spots together with DNSW. For this trial experiment, only two replicates were made for each preceding treatment, together with one blank glass vial with DNSW only and no copepods, resulting in 11 vials. The vials were closed off, making sure no air bubbles remained and the oxygen concentration (μmol / L) was measured every 10 min for a duration of 70 min.

The oxygen concentration measurements showed upward trends in multiple vials, including the control and blank vials, suggesting the test was not performed well, since *N. spinipes species* have an oxygen-consuming metabolism. To find out what went wrong in the process, one of the tests that were performed compared this test method with a test method that included washing the copepods prior to placing them in the vials, excluding any algal feed that could stick onto the species. Unfortunately, after multiple attempts, the cause of unexpected results was not yet found. Further method development, writing down every step of the tests for comparison is in process.

B.5.2 Copepod filtration rate tests

The filtration rate, F (mL / min) quantifies the capacity of copepods to filter food from seawater and is calculated using Equation B.1.

$$F = \frac{[\ln(C_0) - \ln(C_e)] \cdot V}{N \cdot \Delta t} \tag{B.1}$$

In Equation B.1, C_0 and C_e are the algal cell densities (cells / mL) at respectively the start and the end of the exposure duration. V (μL) is the volume in which the copepods are grazing, N (-) the number of copepods, and Δt (min) the exposure duration.

No MPs exposure at 22 °C

In a first trial test, we compared the filtration rate of pre-starved and pre-fed *N. spinipes* at 22 °C without MPs exposure. To avoid transferring algae, larvae or juveniles along with *N. spinipes* adults to the test vessels, forty copepods were first removed from the cultures and put in two groups of twenty in 7 mL of 22 °C DNSW. On a 24-well plate, eight wells were then filled with 2 mL of 22 °C DNSW and five *N. spinipes* adults each. Four of these wells were supplemented with 100 μL of algae suspension (*R. salina*), which resulted in an algal cell density of about $2.5 \cdot 10^5$ cells / mL in each of these wells. These copepods were then incubated in dark at 22 ± 1°C for 24 hours. After 24 hours of starvation or feeding, all copepods were transferred to a new well on the 24-well plate with 2 mL of 22 °C DNSW and they were all fed with *R. salina* at a concentration of about $2 \cdot 10^5$ cells / mL. Four 2 mL control wells containing only $2 \cdot 10^5$ cells / mL *R. salina* in DNSW without copepods

were also made. The algal cell densities in each well were determined with ImageJ software from pictures under a light microscope with 10x objective at the start (0 h) and after 1.5 h and 24 h. From this, the average filtration rates for each treatment (pre-starved copepods and pre-fed copepods) were calculated with Equation B.1. We decided to include one treatment with pre-starved copepods in further filtration rate tests for comparison and would pre-feed the copepods that would undergo MPs exposure.

No MPs exposure at 18 °C, 22 °C and 25 °C

In a second trial test, the method of the first trial test was repeated for three different temperatures at once: 18 °C, 22 °C, and 25 °C. Both the *N. spinipes* cultures and DNSW were incubated for more than 24 h at the according temperature before starting the test. This test can serve as background information for future MPs exposure experiments at different temperatures to evolve to a multiple-stressor approach.

PLGA beads exposure at 22 °C

Before exposing *N. spinipes* to multiple concentrations of PLGA beads, a limit test was performed at 22 °C with an average 5 μm PLGA concentration of $1.7 \cdot 10^4$ # / mL (Appendix B.2).

To prepare this test, 10 mL of 22 °C DNSW and twenty copepods were added to three wells on a 6-well plate. Three treatments were made. One well was filled with only DNSW (pre-starved), one well was filled with DNSW and 360 μL *R. salina* algae suspension (control, pre-fed), and the last well was filled with DNSW, 360 μL *R. salina* suspension and 10 mL of the PLGA solution (pre-fed + MPs exposure). The total volumes in the wells were made to be equal by adding more DNSW where needed. The 6-well plate was put in dark at 22 °C for 48 h prior to the test.

After 48 h, the filtration rate test was set up on a 24-well plate. At the start of this test, 30 mL of new algal suspension with a measured (with FlowCam, Appendix A.1.2) cell density of about $1 \cdot 10^5$ cells / mL was made. Five wells were filled with 2.5 mL of this algae suspension. Five copepods of each treatment were then transferred to one of these wells. This resulted in two wells with algae suspension without copepods and three wells with copepods that were exposed to different treatments. One of the wells without copepods was used to check the algal cell density at the start and the other one at the end of a 2 h incubation in dark at 22 °C.

After the first 2 h incubation, the process was repeated. Another 2.5 mL algae suspension was added to five other wells and the test copepods were transferred

to three of these wells, according to their prior treatment. The algal cell densities in the previously used wells were then measured, as well as the cell density in one of the new copepod-free wells. Again, the plate was incubated for 2 h at 22 °C and afterward, the final algae cell densities were measured.

We repeated further copepod filtration rate tests multiple times, including tests with three different 5 μm PLGA concentrations of $1{\cdot}10^3$, $1{\cdot}10^4$, and $7{\cdot}10^4$ particles / L (Appendix B.2) and calculating filtration rates over different time spans. No clear trends were yet determined considering three replicates each. The test method is currently being followed up and improved.

APPENDIX C

EXACT VALUES

C.1 Algal growth inhibition tests

C.1.1 pH measurements

To check if the algal growth inhibition tests were correctly performed (Section 3.3), the pH of the controls was measured at the start and at the end of the 72 h exposure duration (Table C.1).

Table C.1: Measured pH in the control test vessels of the algal growth inhibition control test with potassium dichromate ($K_2Cr_2O_7$) before and after the 72 h exposure period.

Control replicate	pH start (-)	pH end (-)
R1	7.98	8.19
R2	8.00	8.19
R3	7.99	8.18
R4	8.00	8.20
R5	8.00	8.19
R6	8.00	8.20

C.1.2 Normality test results

To perform one-way ANOVA on the average specific growth rates of different treatments, normality of the replicates within the treatments was checked with a Shapiro-Wilk test (stats package) in R v4.2.3. (R Core Team, 2021). All p-values are given in Table C.2.

C.1.3 Homoscedasticity test results

To perform one-way ANOVA on the average specific growth rates of different treatments, the homoscedasticity of the treatments was checked with a Levene test (car package) in R v4.2.3. for which the null hypothesis says that the variances are equal across all treatments (R Core Team, 2021). All p-values are given in Table C.3.

Table C.2: Shapito-wilk normality test results (p-values) on average specific algal growth rates per concentration (treatment) of *P. tricornutum* exposed to the tested substances in algal growth inhibition tests. Normality was accepted when $p \geq 0.05$.

Test substance	**Concentration**	**p-value**
$K_2Cr_2O_7$	0 mg / L	0.29
	2.5 mg / L	0.64
	5 mg / L	0.41
	10 mg / L	0.99
	20 mg / L	0.99
	40 mg / L	0.90
pristine PVC	0 % v / v	0.07
	90 % v / v	0.71
pristine batch for PLA	0 % v / v	0.31
seawater-aged batch for PLA	0 % v / v	0.99
pristine SR-PLA	10 % v / v	0.67
	24 % v / v	0.05
	60 % v / v	0.31
pristine Flax-PLA	10 % v / v	0.77
	24 % v / v	0.42
	60 % v / v	0.76
seawater-aged SR-PLA	10 % v / v	0.70
	24 % v / v	0.46
	60 % v / v	0.13
seawater-aged Flax-PLA	10 % v / v	0.75
	24 % v / v	0.16
	60 % v / v	0.57

Table C.3: Levene homoscedasticity test results (p-values) on average specific algal growth rates of *P. tricornutum* exposed to the tested substances in algal growth inhibition tests. Homoscedasticity was accepted when $p \geq 0.05$.

Test substance	**p-value**
$K_2Cr_2O_7$	0.44
pristine PVC	0.70
pristine SR-PLA	0.65
pristine Flax-PLA	0.90
seawater-aged SR-PLA	0.49
seawater-aged Flax-PLA	0.55